Integration of Reaction and Product purification for trienzymatic catalyzed synthesis of Laminaribiose from Sucrose

Von der Fakultät für Lebenswissenschaften

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II. Danksagung

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“Without struggle, there can be no progress“

Frederick Douglass (1819-1895)

Zusammenfassung

In der vorliegenden Arbeit wurde Laminaribiose in einem "One-pot Prozess" untersucht. Als Substrat wurde Sucrose verwendet, die enzymatische Synthese erfolgte durch sucrose phosphorylase (SP), glucose isomerase (GI), und laminaribiose phosphorylase (LP). Diese trienzymatische Reaktion dient ebenfalls als Modelsystem für multienzymatische Prozesse. Für eine kontinuierliche Prozessführung wurde die Immobilisierung der Enzyme mit Chitosan-Polyphosphat sowie Agar untersucht.

LP wurde durch *Euglena gracilis* kultiviert und optimiert. Optimale Wachstumsbedingungen für die Produktion wurde durch heterotrophische Kultivierung bei 33°C erreicht, mit Citronensäure-Phosphatpuffer bei pH 4.1. LP konnte nach 96 Stunden durch Ultraschallaufschluss geerntet werden, mit Ausbeuten von 0.52 g/l pro Kulturvolumen. Aufreinigung und Aufkonzentration der LP Enzymsuspension wurde durch Ammoniumsulfatfällung mit 30%-60% Sättigung und anschließender Dialyse erhalten. Aufgereinigtes LP existiert als Monomer und Dimer mit einer Molekularmasse von 120 kDa und 250 kDA.

Optimale Bedingungen für die höchste katalytische Aktivität der LP liegen zwischen 42°C-45°C bei pH 6,2. Die Doppelsubstratkinetik wurde mit den Reaktionen zu den höheren Oligomeren erweitert um ein kinetisches LP Model zu beschreiben. Die Kinetik hängt vom Glucose/Glucose-1-Phosphat Verhältnis ab. Ein Verhältnis größer als 2 verursacht eine Verschiebung der Produkte zu Laminarbiose. Die Bildung höherer Oligomere wird dabei deutlich reduziert. Optimale Reaktionsbedingungen für SP herrschen bei 45°C-50°C und pH 7,0. Das Reaktionsmodel wird durch eine Doppelsubstratkinetik mit Substratinhibierung beschrieben. Für GI sind die optimalen Reaktionsbedingungen bei 60°C und pH 8,5. Die Reaktion folgt einer reversiblen Monosubstratkinetik.

Das trienzymatische System verfügt über optimale Bedingungen bei 45°C mit pH 6,2. Laminaribiosebildungen sind geringer als die Bildung der höheren Oligomere. Die Reaktion wird durch Kombination von Mono-/Bisubstratkinetiken sehr gut beschrieben. Um aufgereinigte Produkte zu erhalten und um die Bildung von höheren Oligomeren zu reduzieren wurde eine reaktionsintegrierte Produktentferung (ISPR, engl. in situt product removal) mit BEA150 als Adsorbens etabliert. Dadurch konnte Laminaribiose annähernd vollständig von der Reaktionslösung entfernt werden.

Die Immobilisierung der Enzyme in einer Chitosan/Polyphosphatmatrix sowie Agarmatrix war erfolgreich, mit guten Enzymaktivitäten. Die Untersuchungen zeigten, dass Enzyme in Agar über eine höhere Aktivität verfügen als in Chitosan/Polyphosphat und daher für die Etablierung eines immobilisierten trienzymatischen/ISPR Systems eingesetzt werden sollten. Die erfolgreiche Produktion von Laminaribiose durch ein immobilisiertes trienzymatischen System konnte bestätigt werden.

Abstract

In this thesis, sucrose is used as a substrate with sucrose phosphorylase (SP), glucose isomerase (GI), and laminaribiose phosphorylase (LP) to produce laminaribiose by establishing a one-pot process. This trienzymatic reaction serves also as a model system for multienzymatic processes. For a continuous process, immobilization with matrix entrapment using chitosan-polyphosphate and agar was studied.

LP was cultivated and optimized using *Euglena gracilis*. Optimal growth conditions for the production are accomplished with a heterotrophic cultivation at 33°C with a citric acid phosphate buffer at pH 4.1. LP is harvested after 96 hours by using ultrasonic disintegration with yields of 0.52 g/l per culture volume. Ammonium sulfate precipitation with 30%-60% saturation, followed by dialysis, is suitable for purification and concentration of LP. The purified LP solution exists as a monomer and dimer with molecular masses of 120 kDa and 250 kDa respectively.

LP was found to have optimal working conditions between 42°C-45°C at pH 6.2. A double substrate mechanism was extended to include the subsequent reactions to higher oligomers as a reaction model for LP kinetics. Kinetics are dependent on glucose/glucose-1-phosphate ratios. Ratios greater than 2 shift the products towards laminaribiose, while greatly reducing higher oligomer formation. Optimal reaction conditions for SP were found at a temperature of 45°C-50°C at pH 7.0. The reaction was modeled with double-substrate kinetics with substrate excess inhibition. Best reaction conditions for GI were found at 60°C-80°C with pH 8.5, the reaction was modeled with reversible monosubstrate kinetics.

With regards to the trienzymatic system, a temperature of 45°C and a pH of 6.2 represent optimal operating conditions. Laminaribiose formation was surpassed by subsequent reactions which lead to the formation laminaritriose and laminaritetraose. The trienzymatic system is well described by combination of mono-/bienzymatic kinetics. To obtain purified products and minimize the side reactions, an in-situ product removal (ISPR) system was established, with BEA 150 used as an adsorbant. Nearly all of laminaribiose was removed from the reaction mixture.

The immobilization of all enzymes within a chitosan/polyphosphate matrix and agar matrix entrapment were both successful showing good enzymatic activities. Comparison of the two immobilization techniques showed that agar is to be favored for establishing an immobilized trienzymatic/ISPR system. A trienzymatic system with the enzymes immobilized in agar showed a production of laminaribiose.

1. Introduction

Carbohydrates are the most important class of organic compounds and represent 75% of the annual regenerative biomass (Lichtentahler and Peters 2004). They appear as mono-, di-, oligo-, and polysaccharides (Lindhorst 2000). Through photosynthesis over 200 billion tons of carbohydrates are produced each year (Lindhorst 2000). They have potential as renewable energy sources, an example being bio ethanol (United Nations Environment Program 2009). Also, they can provide an alternative to fossil fuel plastics, derived from petroleum, namely bioplastics, which can also be obtained from corn or pea starch (CORDIS 2008).

Carbohydrates have started to become increasingly valuable for several industrial fields. For example, biodiesel fuel uses energy from a carbohydrate source (Demirbas 2009). In 2010, the worldwide biofuel production reached 105 billion liters (Worldwatch Institute 2010). Bioplastics, obtained from corn or peach starch, which are renewable biomasses, can be applied for disposable items, such as packaging, or non-disposable, an example being car interiors, fuel line and plastic pipe applications (www.microtecco.com, CORDIS 2008). Particularly interesting are the uses of higher oligosaccharides in the pharmaceutical industry. They are utilized for the treatment of dermatitis (Sugiyama et al. 2010). The application as a vaccine against cancer is another possibility for functionalized sugars (Buskas 2005). In terms of food and nutrition applications, the key word would be ´functional food´. Functional food describes a dietary ingredient which provides additional health-promoting and disease preventing functions and is provided by many oligosaccharides (Gibson and Roberfroid 1995). The most important approaches for dietary modulation in the human body are probiotics, prebiotics and omega-3-fatty acids.

- Probiotics are foods sources containing specific microorganisms, which positively influence the host organism (FAO/WHO 2001). Examples of probiotic bacteria are *Bifidobacterium animalis* in Danone products and *Lactobacillus johnsonii* in Nestlé products.
- Prebiotics are non-digestible food ingredients promoting growth and activity of bacteria in the digestive system (Gibson and Roberfroid 1995), an example being fructooligosaccharides.
- Omega-3-fatty acids count as an essential substance for human diet, which the body, however, is not able to synthesize itself (AHRQ 2005), an example being linoleic acid.

The classical chemical approach to produce functionalized carbohydrates usually consists of multi-step reactions and includes mostly blocking group chemistry since carbohydrates often do not react selectively (Hanessian 1997). Often the intermediates have to be isolated and purified before they can be used as substrates for the following reaction step, leading to low yields, high production and downstream processing costs(Buchholz et al. 2012). Therefore the industry focuses more and more on

biocatalytical reactions (Antranikian 2006). As a result, the number of processing steps can be reduced and higher yields can be obtained with lower costs and waste amounts, using less resources and energy (Flaschel et al. 2004). Biocatalysis is an excellent alternative to conventional chemical methods, especially for the formation of complex structures. The main application can be found in the fine chemicals industry, with 4-5% of the involved processes relying on enzymes, with a rising tendency (Antranikian 2006).

As biocatalytical processes begin to replace classical chemical methods, the chemical process engineering, alongside with new biotechnological development must be taken into consideration. It is very complex to improve a biocatalytical procedure on a larger scale (Tufvesson et al. 2009). "Frequently solutions necessitate a compromise between different requirements and therefore good analytical and design tools are required to evaluate the many choices that are presented, to avoid running into dead-ends" (Tufvesson et al. 2009).

As of today, mainly single enzymes are applied on a technical scale, both in native and immobilized form (Santacoloma 2011). However, multienzymatic systems have also been investigated (Findrik and Vasic-Racki 2009, Jördening et al. 2008, Santacoloma 2011). As each enzyme has its own specific pH and temperature level to gain high activity (Erhardt et al. 2008), multienzymatic systems become more complex and make it necessary to find conditions under which all enzymes will retain their activity when combined in a single reactor.

In this thesis, three enzymes are used in a trienzymatic system to produce laminaribiose, using the cheap substrate sucrose. This system serves as a model reactor not only for the sugar itself but for general multienzymatic reactions.

2. Objective

The work can be divided into several research topics:

A method is to be established to produce laminaribiose, using one of the cheapest sugars available on the market, sucrose. A trienzymatic system ought to be created, which converts sucrose to laminaribiose (figure 2.1). Sucrose phosphorylase (SP) catalyzes the transfer of glucose from sucrose to phosphate, yielding glucose-1-phosphate and fructose (Silverstein et al. 1967). Glucose isomerase (GI) is used to isomerize fructose to glucose (Bhosale et al. 1996), so that glucose does not have to be additionally provided to the system. The third step is the laminaribiose phosphorylase (LP)-catalyzed glucosidation of glucose with glucose-1-phosphate yielding laminaribiose (Goldemberg et al. 1966).

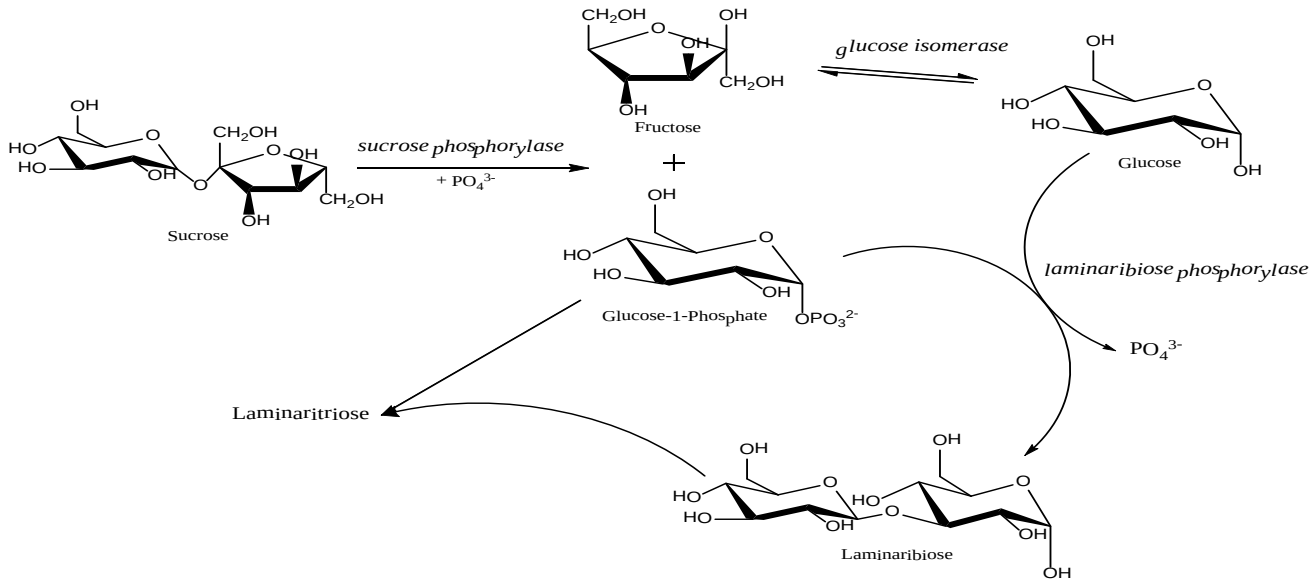


Fig.2.1 Reaction scheme for the production of laminaribiose

The following aspects are necessary to research and optimize

- Microbial production, characterization and optimization of LP
- Kinetic characterization and the determination of optimal operating conditions for SP and GI
- Operation of a native trienzymatic and in-situ product removal (ISPR) system
- Immobilization and characterization of each enzyme
- Operation of an immobilized trienzymatic and ISPR system

Whereas SP and GI are commercially available, LP needs to be obtained from *Euglena gracilis*. Hence different methods for the cultivation of the cells and production of LP are studied to optimize the production of the enzyme.

Using established methods, each enzyme is characterized by the kinetic parameters and typical reaction parameters such as temperature, pH and stability. Based on those results, the enzymes are combined in a trienzymatic system, to find optimal parameters for the production of laminaribiose.

To realize holding the enzymes in place throughout the reaction, following an easy separation from the products and re-utilization of the biocatalysts, immobilization in a chitosan-polyphosphate complex and a matrix entrapment in agar is studied. When successful, a system with the combination of all of the immobilized biocatalysts needs to be found and optimized. For an application to lab and pilot scale, with a reaction integrated separation of the product, a modeling of the entire system is required.

Thus, this trienzymatic system serves as a source for laminaribiose synthesis as well as a general model system for multienzymatic reactions.

3. Theoretical Background

Carbohydrates play a central role in life and have many applications for living organisms (Maton 1993). Polysaccharides, like starch, produced by all green plants, are storage for energy and serve as structural components (cellulose) (Lehmann 1996). Deoxyribose and ribose are the backbones of the genetic molecules DNA and RNA (Berg et al. 2012). Furthermore saccharides and their derivatives have key roles in the metabolism, needed for example in the immune system, fertilization and blood clotting (Koolman 2003).They are also important in the metabolism, in which saccharides/chemical substances are converted into materials needed for the functionality of the human being (Koolman 2003).

Without the help of certain biological molecules which speed up the reactions, it would basically be impossible to use carbohydrates and other substances in metabolic processes (Smith 1997). This is where enzymes come into play. In a biological cell, nearly all chemical reactions rely on enzymes in order to occur at rates needed to be life sustaining (Koolman 2003). Since enzymes are selective to their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathway occurs in that cell (Renneberg 2007).

Enzymes and carbohydrates received a rising attention in the past decades, because of their ability to be combined for the formation of new products, while keeping production costs relatively low (Antranikian 2006). The most famous example is the production of „high-fructose-corn-syrup“(Guzmán-Maldonado and Paredes-López 1995).

3.1 Carbohydrates: fuel for the body

Foods containing carbohydrates include fruits, beans, potatoes, bread, and cereals (Loderbauer 2013). It is well known that carbohydrates are an important source of energy, but they are not considered as essential nutrients for the human body (Westman 2002, Shah and Pandya 2013). Although many organisms have the ability to metabolize other monosaccharides and disaccharides, glucose is preferred (Siegel 1999).

Energy sources are the digested nutrients, which are enzymatically broken down via specific metabolical reaction paths (Lienhard et al. 1992). In addition, small molecules are formed from which the building blocks of the cells are built, e.g.: glucose, amino acids, nucleotides, organic acids and lipids (Renneberg 2007).

The carbohydrates of relevance for this thesis are discussed below.

3.1.1 Sucrose

Sucrose (Suc) is a white odorless, crystalline compound with a sweet taste (Mintz 1986). It is mainly used as a sweetener with an consumption per person of 35 kg/a in 1991 (Vollhardt 2000). The origin of sugar used as a sweetener is not yet known. It is believed that over 5000 years ago the sugar cane was first cultivated and utilized in the Polynesian islands (www.sugarnutrition.org.uk), other sources place its origins in India (Rolph 1917). Nonetheless the cultivation of the sugar cane quickly spread to China, where the crystallized table sugar as we know it was refined (Parker 2011). In the mid 18th century, the German chemist Andreas Marggraf isolated sucrose in sugar beet (Marggraf 1747). The discovery of the sugar beet only had little importance for the commercial manufacturing industry for half a century (Rolph 1917). This rapidly changed after the Napoleonic Wars in which the import of sugar from sugar cane was prohibited (Rolph 1917). As a consequence, the beet sugar industry grew larger and eventually replaced sugar from the sugar cane. In 2011, 168 million metric tons of sucrose was produced worldwide (USDA 2011).

The disaccharide sucrose is composed of α-D-glucose and β-D-fructose linked with α,β-1.2-glycosidic bond (figure 3.1) (Vollhardt 2000). It contains no anomeric hydroxyl groups and is classified as a non-reducing sugar (Cox and Lehninger 2011).

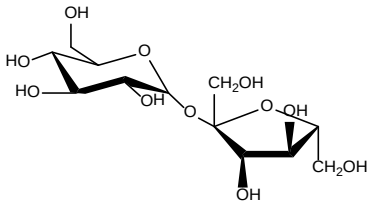


Fig.3.1 Chair conformation of sucrose

By usage of a sucrase within the small intestine, sucrose is cleaved into glucose and fructose, hence quickly providing energy. (Koolman 2003).

Besides its cheapness in terms of production, the main importance of sucrose in this thesis is its glycosidic bond. The glycosidic bond is formed between the anomeric hydroxyl group of glucose and the hydroxyl group of the C2 atom of the fructose unit (Vollhardt 2000). The glycosidic bond provides energy for the glycosylation reaction (Vollhardt 2000).

3.1.2 Glucose/Fructose

Glucose (Glc) represents the main carbohydrate energy source for most organisms and it is being used by cells as metabolic intermediate (Renneberg 2007, Siegel 1999) It enters the bloodstream , where it is delivered to the entire body (Lienhard et al. 1992).

When not in solution, glucose exists as an hexoaldose in an open chain form (figure 3.2) (Vollhardt 2000). When placed in an aqueous solution, the glucose forms a hemiacetal pyranose structure, with the open-chain form being limited to 0.20 % (figure 3.2) (Vollhardt 2000).

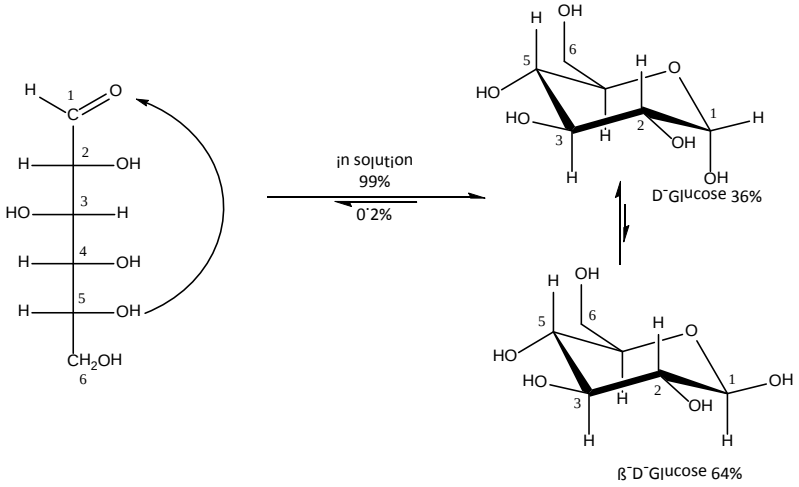


Fig.3.2 Glucose structural behavior in solution (from Vollhardt 2000)

Fructose is a monosaccharide in the form of a ketohexose and has an open-chain form when crystalline (Vollhardt 2000). Ones placed in solution, fructose forms a cyclic hemiacetal, occurring as an equilibrium mixture of fructopyranose (70%) and fructofuranose (30%) (Vollhardt 2000).

3.1.3 Glucose-1-phosphate

Cori-Ester, describes a glucose molecule containing a phosphate group on the C1-atom (figure 3.3) (Wolfrom 1942).

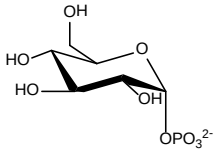


Fig.3.3 Structure of glucose-1-phosphate

It has a major role in terms of initiating the carbohydrate mechanism (Wolfrom 1942).

Phosphoglucomutase catalyzes the reaction of glycogen and inorganic phosphate to eventually yield glucose-1-phosphate (Sutherland et al. 1941). G1P is isomerized into glucose-6-phosphate (G6P) (Sutherland et al. 1941), a molecule with a major role in the pentose phosphate cycle and glycogen synthesis (Berg et al. 2012).

3.1.4 Laminaribiose

An interesting type of ß-1.3-linked glycosides is laminaribiose (Lam) (3-O-(ß-D-glucopyranosyl)-D-glucose) (figure 3.4), which can be obtained from hydrolysis of laminarin extracted from natural polysaccharides of plant origin (Villa et al. 1978).

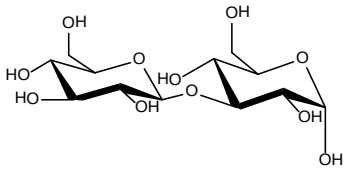


Fig.3.4 Structure of laminaribiose

In this method, ß-1.3-glucanases depolymerise ß-1.3 glucanes, such as laminarin, to obtain smaller fragments including laminaribiose (Giese et al. 2006). However, it is an uneconomical process, as the unwanted oligosaccharide fragments and monosaccharide products must be removed from the mixture to obtain relatively pure laminaribiose (Kitaoka et al. 1993). Also, laminarin as a substrate is relatively expensive (Kitaoka et al. 1993). It can also be obtained by thermal condensation of glucose by heating the sugar at 150°C (Sugisawa and Edo 1966). In this reaction, laminaribiose was produced alongside nigerose, sepharose and others (Sugisawa and Edo 1966). The mechanism is not yet fully explained, but it is known that the glucosidic hydroxyl group reacts with the alcoholic hydroxyl group of another glucose molecule (Sugisawa and Edo 1966).

Laminaribiose itself shows promising applications in the pharmaceutical industry as building blocks (Bader et al. 1996). In its peracetylated form, laminaribiose is useful as a starting material for many organic syntheses (Wang et al. 1991). It possesses prebiotics properties, giving it potential in the food industry (Sanz et al. 2005).

The main topic of this thesis is to produce laminaribiose using a trienzymatic system (Chapter 2). Sucrose is utilized with the application of sucrose phosphorylase, glucose isomerase, and laminaribiose phosphorylase to produce laminaribiose (ß-1.3-diglucoside) in a one-pot process. As opposed of using α-glucose-1-phosphate and glucose as starting materials, the trienzymatic process is a more promising progression (Kitaoka et al. 1993). Sucrose has a distinct advantage over glucose-1-phosphate in terms of availability and cost efficiency. With sucrose, a cheap substrate is available and highly suitable to be used for a low-cost production of laminaribiose. Also relatively few purification steps are necessary as opposed to laminarin hydrolysis and thermal condensation of glucose.

3.1.5 Higher Oligomers (laminaritriose/laminaritetraose/etc…)

An oligosaccharide describes a saccharide polymer containing typically three to six units of monosaccharides (Encyclopaedia Britannica). Oligosaccharides possess many biological functions in organisms, such as structural roles, modulations of protein functions, and cell-cell recognition (Varki 1993). Oligosaccharides, such as human milk oligosaccharides, serve as a food source for the intestinal microflora, causing prebiotic effects (Bode 2009). Based on clinical studies, oligosaccharides such as fructooligosaccharides can increase the number of helpful bacteria in the colon, while the harmful ones are reduced (Macfarlane et al. 2007).

With regards to the objective of this thesis, tri-, tetra-, and pentasaccharides will be formed alongside laminaribiose during the enzymatic reaction process using laminaribiose phosphorylase (Goldemberg et al. 1966) (figure 3.5). They are however regarded as undesired side products (see also 3.2.1.2).

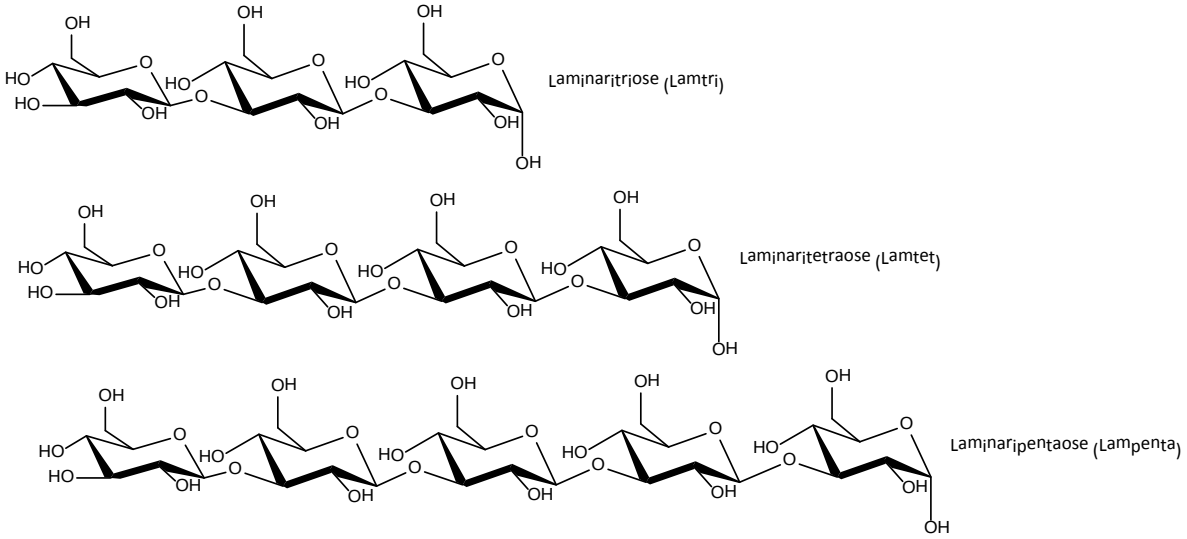


Fig.3.5 Higher Oligosaccharides formed during enzymatic production of laminaribiose

3.2 Enzymes for the trienzymatic system

3.2.1 Glycosyltransferases

As seen above, carbohydrates and polysaccharides are of biological importance. They carry out many important tasks, like protein glycosylation and energy storage, as well as cell-cell interactions and signaling (Rudd et al. 2001; Wells et al. 2001; Bertossi and Kiessling 2001). Because of these interacting and signaling functions, they can be seen as a cellular language and rely on specific carbohydrate structures (Coutinho 2003). The biosynthesis of those ´specialized´ molecules is realized by glycosyltransferases (Coutinho 2003). Glycosyltransferases (EC 2.4.x) are a specific group of enzymes which catalyze the transfer of a carbohydrate from an activated sugar donor onto saccharide or non-saccharide acceptor by a glycosidic bond, occurring either with retention or conversion of the donor´s anomeric center (figure 3.6) (Kleene and Berger 1993, Coutinho 2003). Although they catalyze chemically similar reactions, they show a huge diversity in terms of donor, acceptor and product specifity (Coutinho 2003).

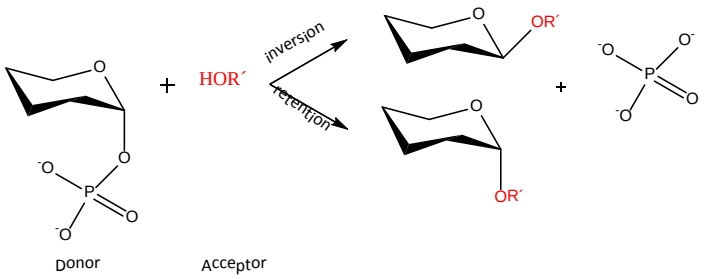


Fig.3.6 Glycosyltransferases use sugar donors, in this case G1P (OH groups taken out for better overview), in which a carbohydrate is transferred onto an acceptor. The acceptor is shown in red. Donor´s anomeric center can be inversed and retained (from Coutinho 2003).

Depending on their substrate specifity, glycosyltransferases can be classified as either Leloir or non-Leloir transferases (Leloir 1971).

Leloir transferases use nucleotide activated saccharides (e.g. UDP-glucose) as substrates (figure 3.7). They are highly regio- and stereoselective and only tolerate minor substrate/donor modifications (Seibel et al. 2006).

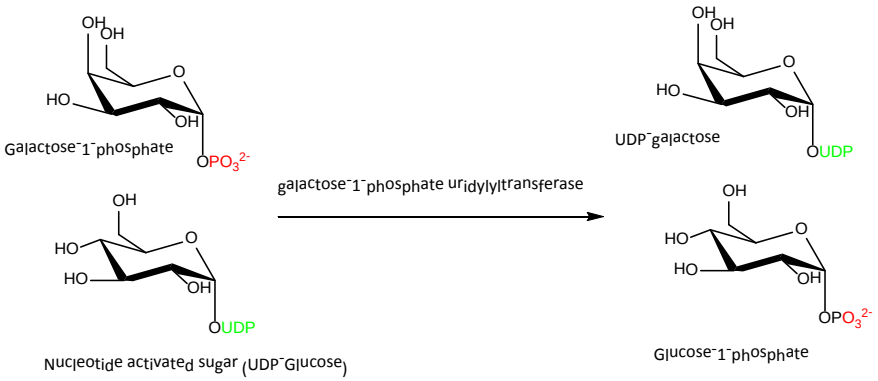


Fig.3.7 Glycosylation of a nucleotide activated polysaccharide UDP-glucose and galactose-1-phospate with a Leloir transferase (Holden 2003)

The applications of these type of glycosyltransferases on industrial scale are limited, due to the high costs of nucleotide-activated substances, thus non-Leloir transferases have been applied for industrial usage (Seibel et al. 2006).

These enzymes use non-nucleotide activated saccharides, whose glycosidic bonds provide the required energy for the reaction, for the transfer of the glycosyl group (figure 3.8) (Seibel et al. 2006).

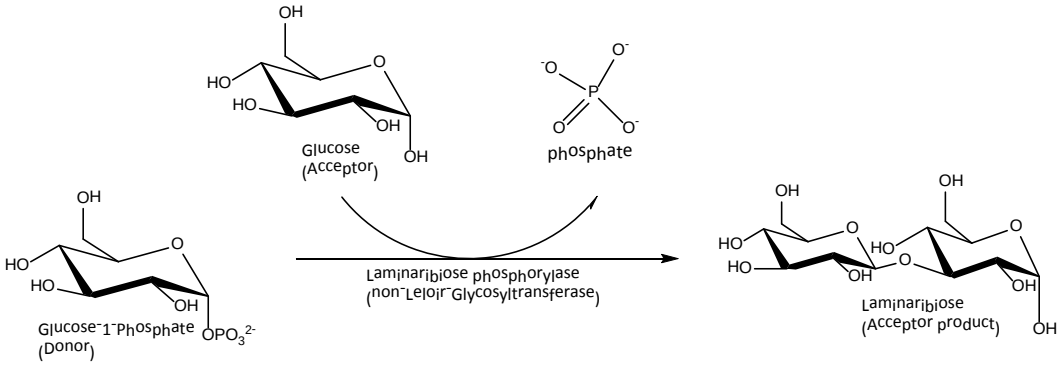


Fig.3.8 Reaction scheme for the production of laminaribiose with laminaribiose phosphorylase (Goldemberg et al. 1966)

For this thesis two glycosyltransferases, sucrose phosphorylase and laminaribiose phosphorylase, are used.

3.2.1.1 Sucrose phosphorylase (EC 2.4.1.7)

Sucrose phosphorylase (SP) from *Leuconostoc mesenteroides* catalyzes the glycosyl transfer to phosphate by using sucrose as a donor substrate (Luley-Goedl and Nidetzky 2010).

The enzyme possesses 488 amino acids and its molecular weight is 55 kDA (Koga et al. 1991). SP is able to catalyze three different reaction types, being *hydrolysis*, with a water site at its active center, as well as *phosphorolysis and transglucosylation*, with the active center having an acceptor site (Schwarz and Nidetzky 2006, Luley-Goedl et al. 2010).

It has been confirmed that the reaction is characterized by a double displacement mechanism with retention of the anomeric configuration of the donor (also referred to as “ping-pong” mechanism), to convert its disaccharide substrate (sucrose) and phosphate into glucose-1-phosphate and fructose, as initially suggested by Cleland (Cleland 1963, Mieyal and Abeles 1970, Luley-Goedl et a. 2010) (figure 3.9).

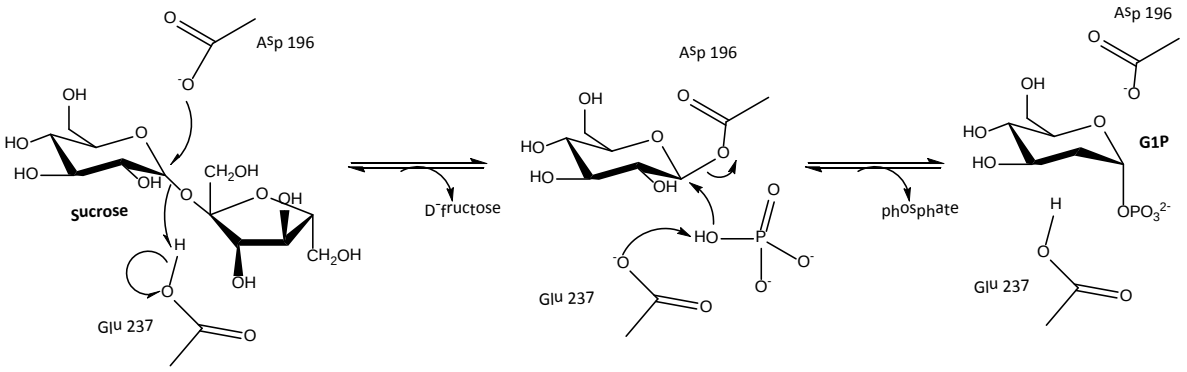


Fig.3.9 Reaction mechanism for sucrose phosphorylase (Luley-Goedl and Nidetzky 2010)

The reaction of the transglucosidation is initiated by the binding of sucrose to the active site of the enzyme, releasing fructose and leaving a covalent glucosyl-enzyme-complex (Luley-Goedl and Nidetzky 2010). The complex consists of a ß-linkage between the C1 atom of the glucosyl-residue and the

oxygen of the carboxylic group (Mirza et al. 2006). A phosphate group cleaves the glycosidic bond, resulting in the formation of α-glucose-1-phosphate (Luley-Goedl and Nidetzky 2010).

Crystal structures from SP strongly support the mechanism as proposed in figure 3.9 and reveal the Asp^{196} as the catalytic nucleophile "attacking C-1 of the glucosyl moiety of sucrose" (Goedl et al. 2008, Schwarz and Nidetzky 2006).The reaction mechanism requires a Bronsted catalyst, which appears in the form of Glu^{237} (Schwarz et al. 2007). It is capable of "donating its proton to the dislocated hydroxyl group to the glucosides C-1 atom" (Schwarz et al. 2007). During cleavage, the resulting glucose binds covalently to the enzyme. The phosphorylation of the glucosyl residue forms a C-1 carbenium ion, which promotes the break of the ester bond between the glucosyl residue and Asp^{196} (Schwarz and Nidetzky 2006).

3.2.1.2 Laminaribiose Phosphorylase (EC 2.4.1.31)

Laminaribiose phosphorylase (LP) from *Euglena gracilis* is a carbohydrate phosphorolyzing enzyme, more specifically a ß-1.3-oligoglucan orthophosphate glucosyltransferase, which reversibly phosphorolyzes glycosidic bonds to produce monosaccharide 1-phosphates at the reducing end of the molecules (Kitaoka et al. 1993b, Goldemberg et al. 1966) (figure 3.10). Instead of phosphate, other acceptors such as cellobiose or ß-phenolglucosides can also be utilized (Goldemberg et al. 1966).

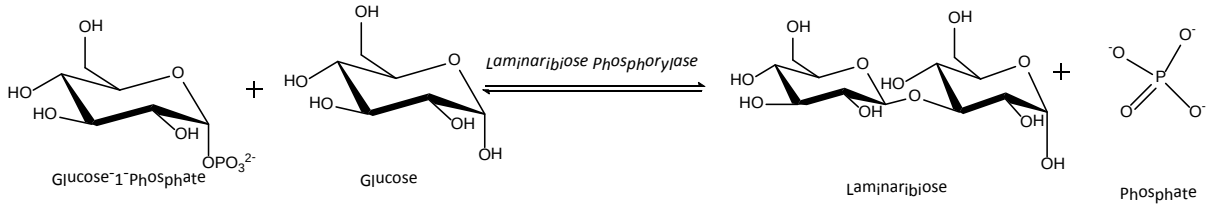


Fig.3.10 Reaction scheme for production of laminaribiose with LP

This enzyme occurs as an intracellular enzyme in algae and euglenida cells (Kitaoka et al. 1993b). In these cells the enzyme´s function is believed to catalyze the formation of the polysaccharide ß-1.3-glucan (paramylon), used as an energy reserve (Vogel and Barber 1968). It has a molecular mass of 120 kDa and 240 kDa for its dimer form on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) (Kitaoka et al. 1993b).

The enzyme catalyzes an ordered bi-bi-mechanism (Kitaoka et al. 1993b). The kinetic determination of LP is complicated, as laminaribiose phosphorylase also phosphorolyzes the tri-, tetra-, and pentasaccharides (eq. 3.1-3.4) (Goldemberg et al. 1966).

$$\mathbf{Laminaribiose + Phosphate \rightleftharpoons Glucose + G1P} \quad \textbf{(3.1)}$$

$$\mathbf{Laminaritriose + Phosphate \rightleftharpoons Laminaribiose + G1P} \quad \textbf{(3.2)}$$

$$\mathbf{Laminaritetraose + Phosphate \rightleftharpoons Laminaritriose + G1P} \quad \textbf{(3.3)}$$

$$\textbf{Laminaritpentaose} + \textbf{Phosphate} \rightleftharpoons \textbf{Laminaritetraose} + \textbf{G1P} \quad \textbf{(3.4)}$$

Further studies revealed a competitive inhibition caused by glucose (Kitaoka et al. 1993b).

The resulting higher oligosaccharides can be used as a ´functional food´ as well as in the medical field, as in the treatment of dermatitis (Sugiyama et al. 2010).

3.2.2 Glucose Isomerase (EC 5.3.1.5)

In the food industry, the isomerization of glucose to fructose with glucose isomerase is a widely used processes (Dehkordi et al. 2009). “The use of glucose isomerase represents by far the largest application of immobilized enzymes, and accordingly a large number of commercial products have appeared on the market” (Jorgensen et al. 1988). The annual world-wide production of glucose isomerase is around 100000 tons . It is mainly used to industrially manufacture high-fructose-corn-syrup (HFS), a process in which glucose is turned into fructose to produce a desired sweetness (Wallinga et al. 2009).

HFS is composed of glucose and fructose (1:1) in equilibrium), which is 1.3 and 1.7 times sweeter than sucrose and glucose, respectively (Bhosale et al, 1996, Hanover and White 1993). In the United States, foods and products typically use HFS as a sweetener and replaced sucrose (Wallinga et al. 2009).

The product is added into dietary foods and drinks, since it improves "sweetening, color, and reduces viscosity" (Dehkordi et al. 2009). The first glucose-isomerizing enzymes were discovered in 1957 in *Pseudomonas hydrophila* and was at the same time the starting point for exploiting the enzyme in the industry to manufacture a sugar substitute (Marshall and Kooi 1957). Application on industrial scale was successfully implemented by Clinton Corn Processing Co. in the U.S. during 1967 (Bhosale et al. 1996).

In this thesis, GI from *Streptomyces murinus* is used to isomerize fructose to glucose (figure 3.11). GI from *S. murinus* is relatively heat stable between 60°C-80°C (Bhosale et al. 1996) and contains Mg ions as co-factors in its structure (figure 3.13) (Jorgensen et al. 1988).

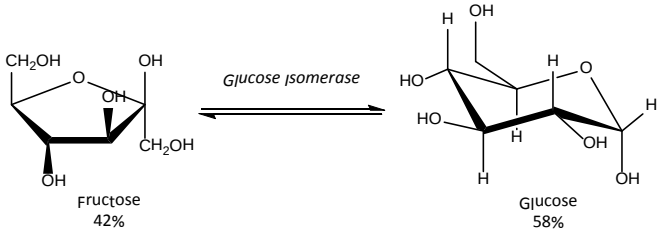


Fig.3.11 Reaction scheme for the isomerization of fructose to glucose using GI

Glucose isomerase interconverts aldoses and ketoses in a reversible reaction (Katz et al. 2006, Bhosale et al. 1996). The conversion is equilibrium controlled and currently economically limited to 42% fructose (Zhang et al. 2004).

Besides its wide-spread application in the food industry, the mechanism and structure of GI are not yet fully understood (Bhosale et al. 1996). It was first assumed to follow an ene-diol mechanism (Rose et al. 1969). However later studies implied a hydride shift mechanism (figure 3.12) (Bhosale et al. 1996). Glucose isomerase enables a ring opening of the substrate (Katz et al. 2006). The opening into the straight chain form is performed close to the Mg ions, represented in figure 3.13 as M1, by participation of His 54 (Katz et al. 2006). The lower region in figure 3.12 is responsible for the isomerization by the hydride shift mechanism from the C2 to the C1 atom, eventually leading to ring closure (Katz et al. 2006, Collyer and Blow 1990).

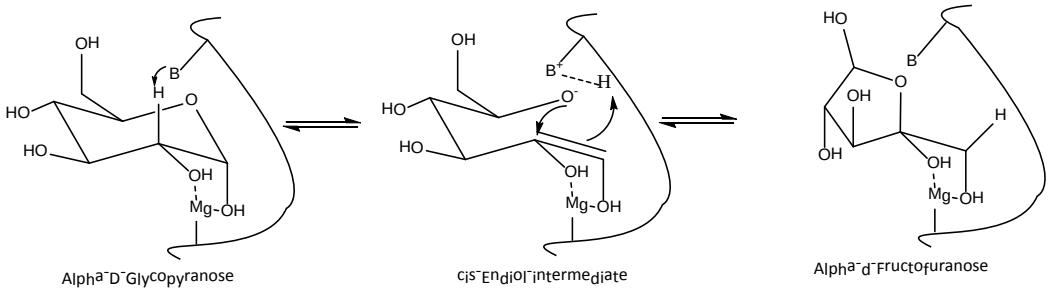


Fig.3.12 Hydride shift mechanism of action of GI (based on Bhosale et al. 1996)

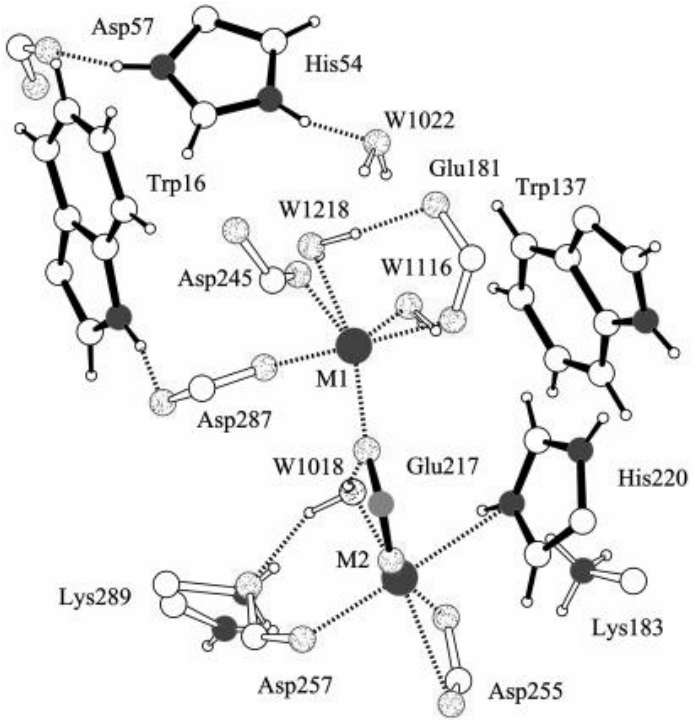


Fig.3.13 Active site of GI with His54 and Glu217 participating in the hydride shift mechanism (Katz et al. 2006)

The molecular weight of GI varies from 52 kDa-191 kDa, depending on the host organism (Bhosale et al. 1996). GI appears as a tetramer or dimer and consists of subunits which are neither covalently bonded nor contain interchain disulfide bonds (Bhosale et al. 1996).

3.3 Enzyme kinetics and its parameters

Enzyme kinetic theory is an important aspect in terms of understanding the reaction involving the enzyme as well as its mechanism and what factor influence the enzyme´s activity.

Enzymes are catalysts that greatly increase the reaction rate by lowering the activation energy of the reaction (figure 3.14) (Chang 2004). The following characteristics for catalysis are to be noticed (Chang 2004).

- "Catalyst provides an alternate path for the reaction "
- "Catalyst does not appear in overall reaction"
- "Catalyst cannot affect the thermodynamic equilibrium constant and are not changed by the reaction"

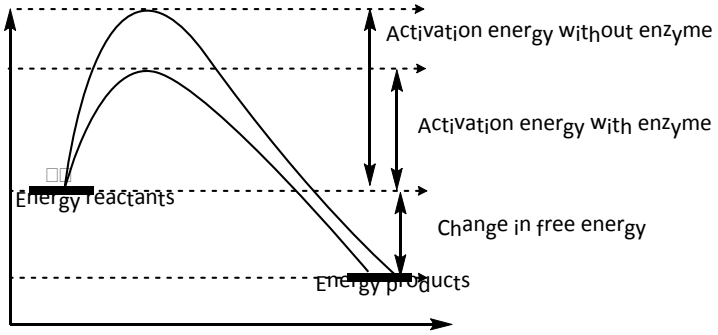


Fig.3.14 Activation energy of a reaction with/without use of an enzyme

3.3.1 Factors affecting the enzyme

The efficiency of an enzymatic reaction depends on temperature, pH, enzyme concentration, substrate concentration, and presence of inhibitors and co-factors (Berg et al. 2012).

3.3.1.1 Temperature

Every enzyme has a specific temperature optimum (Koolman 2003). A rise in temperature causes an acceleration of enzymatic and non-enzymatic reactions, by which the reaction rate will increase until a maximum at a specific temperature is reached (figure 3.15) (Koolman 2003). Increasing the temperature further, leads to an (ir)reversible destruction of the enzyme´s tertiary structure, causing a denaturation (figure 3.15) (Voet et al. 2002).

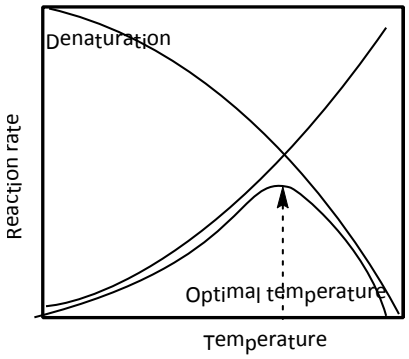


Fig.3.15 Enzyme dependency on temperature

3.3.1.2 pH

Similar to an enzyme having a temperature optimum, each enzyme also possesses a characteristic pH-optimum (Koolman 2003). If the pH of the reaction takes place outside this optimum, the enzyme´s activity is reduced (figure 3.16) (Koolman 2003). This is based on the change in the tertiary structure of the enzyme, caused by the change of the electrical charge of the side chains on the active site (Bisswanger 2000).

This would result in a conformation change, which influences enzymatic activity (Bisswanger 2000).

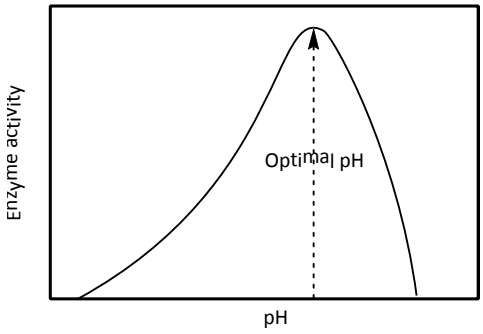


Fig.3.16 Enzyme dependency on pH

3.3.1.3 Inhibition

Inhibitors are substances able to decrease an enzymatic reaction rate, by hindering the enzyme the enzymes activity (Chang 2004). This effect can either be reversible or irreversible (Chang 2004).

1) Reversible inhibition

a) Competitive inhibition

In this type of inhibition, both substrate and inhibitor can bind onto the active site and are in competition to each other (Chang 2004, Berg et al. 2012). In order for the enzyme to overcome this effect, it requires high substrate concentrations (Chang 2004, Berg et al. 2012).

b) Non-competitive inhibition

In this case, the inhibitor is able bind onto another side of the enzyme, without blocking the active site, (Chang 2004). The binding can occur at the free enzyme or enzyme-substrate complex while the substrate is not affected by the inhibitor (Chang 2004, Berg et al. 2012) As a result, the substrate concentration does not have any influence on overcoming the non-competitive inhibition (Chang 2004).

c) Uncompetitive inhibition

The uncompetitive inhibitor is similar to the non-competitive inhibitor, but binds merely to the enzyme-substrate complex (Chang 2004, Berg et al. 2012). Again the active site of the enzyme remains unaffected (Chang 2004).

2) Irreversible Inhibition

The inhibitor and the enzyme form a covalent bond, by which substrate conversion is being blocked, causing an irreversible inhibition (Chang 2004). The deactivation of the enzyme takes place by the covalently modified amino acids side chains of the active site (Berg et al. 2012).

3.3.2 Single-substrate kinetics

For a one substrate reaction, the enzymatic reaction rate can be described with the Michaelis-Menten approach (M-M kinetics) (Michaelis and Menten 1913). In this model (figure 3.17), the enzyme (E) and the substrate (S) form an enzyme-substrate complex (ES) (Chang 2004). In the final step, the enzyme-substrate complex is transformed into the product (P) and the enzyme (Chang 2004).

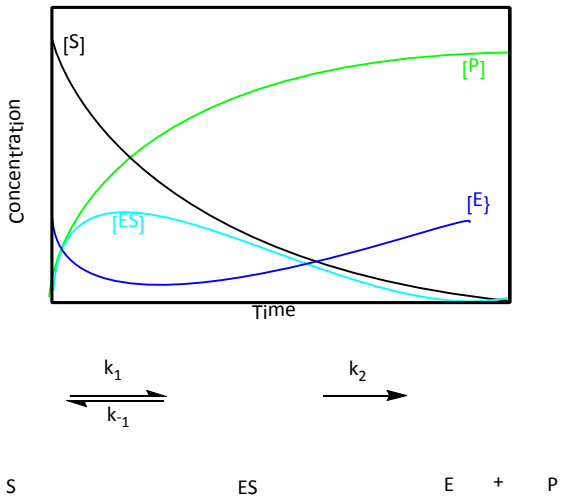


Fig.3.17 Enzymatic reaction model using M-M kinetics

In the original M-M model, it was assumed that the formation of ES can be regarded as a rapid equilibrium process, in which k_{-1} (unimolecular dissociation rate constant to form E and S) is significantly faster than k_2 (unimolecular dissociation rate constant to form E and P)(Michaelis and

Menten 1913, Chang 2004, http://depts.washington.edu/wmatkins/kinetics/michaelis-menten.html). In 1925, George Briggs and J.B.S Haldane demonstrated, that the assumption of a thermodynamic equilibrium is unnecessary (Briggs and Haldane 1925, Chang 2004).

They proposed a steady-state approximation, in which the concentration of the enzyme-substrate complex will quickly take a constant value, after initializing of the reaction (eq. 3.5) (Chang 2004).

$$\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad \textbf{(3.5)}$$

Solving for eq. 3.5, given that $\frac{dP}{dt} = k_2[ES]$ and $[E_0] = [E] + [ES]$, leads to the following basic mathematical equation of enzyme kinetics (eq. 3.6) (Chang 2004, Berg et al. 2012, Koolman 2003).

$$\mathbf{v} = \frac{\mathbf{v_{max}}\cdot[\mathbf{S}]}{\mathbf{K_M}+[\mathbf{S}]} \quad \textbf{with } \mathbf{K_M} = \frac{\mathbf{k_2}+\mathbf{k_{-1}}}{\mathbf{k_1}} \qquad \textbf{(3.6)}$$

K_M describes the Michaelis-Menten-constant, representing the substrate concentration at half-maximum reaction rate (Berg et al. 2012). K_M is a measure for the affinity of an enzyme towards the substrate. The higher K_M, the smaller the affinity and a higher substrate concentration is needed to reach the half-maximum reaction rate (Berg et al. 2012). v describes the current reaction rate of the enzymatic reaction, while v_{max} represents the maximum reaction rate (Berg et al. 2012).

At large substrate concentrations, K_M can be neglected and the reaction rate takes the form of $v = v_{max} = k_2[E_0]$ (0. order reaction) (Chang 2004).In this case, all active site of the enzymes are occupied with substrates. At very low substrate concentrations, [S] in the denominator is neglected and eq. 3.6 takes the form $v = k_2 \frac{[E_0]}{K_M}[S]$ (1st order reaction) (Chang 2004).

The relationship between the reaction rate and the substrate concentration is represented in figure 3.18.

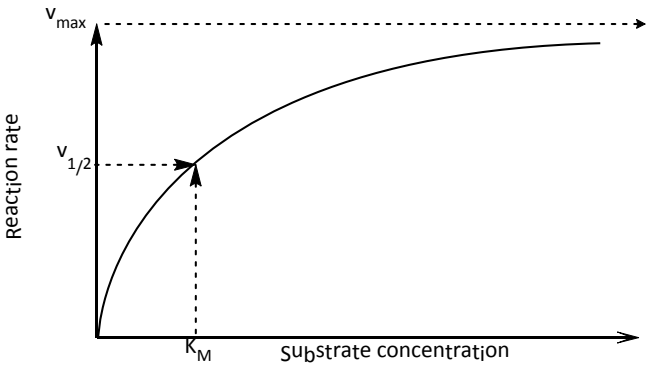


Fig.3.18 Saturation curve of an enzymatic reaction

3.3.3 Kinetics of double-substrate catalyzed reactions

„For reactions with two or more substrates or products, however, the rate equations are much more complex and cannot be explained in terms only of maximum rates and simple Michaelis constants“ (Cleland 1963). In terms of two substrates binding and the products leaving the active site, many

variations of the reaction are possible (Chang 2004). The most common reactions include the binding of one substrate on the enzyme before the other (ordered bi-bi), the binding of the substrates in any order (random sequential), and finally the binding of one substrate, which modifies the enzyme and then leaves to allow another substrate to bind (double displacement) (Chang 2004). These different mechanisms consist of many different reaction rates.

A generalized rate law for modeling two substrate/product enzyme reaction is suggested in eq. 3.7 (Liese et al. 2011). The kinetic models created in this thesis for SP and LP are based on these simplified two-substrate reaction kinetics.

$$\mathbf{v} = \mathbf{v_{max}} \cdot \frac{[\mathbf{A}]}{\mathbf{K_{MA}}+[\mathbf{A}]} \cdot \frac{[\mathbf{B}]}{\mathbf{K_{MB}}+[\mathbf{B}]} \qquad \textbf{(3.7)}$$

[A]: concentration of substrate A
[B]: concentration of substrate B
K_{MA}: M-M-constant for substrate A
K_{MB}: M-M-constant for substrate B

Cleland created a standardized way of bisubstrate (bi-bi) enzymatic reactions, which make up 60% of all enzymatic transformations (Cleland 1963). He described two major mechanisms involved.

3.3.3.1 Sequential bi-bi

This type of mechanism can be further classified into an **ordered** and **random sequential mechanism** (Nelson and Cox 2009). In the **ordered sequential bi-bi**, substrate A must first go onto the active site to create an EA complex, before substrate B can be attached to the enzyme, forming an EAB complex (Chang 2004, Voet et al 2002). EAB is transformed into a PQE complex (Chang 2004, Voet et al. 2002). As a result, product P must leave the active site before the second product Q can be released (Chang 2004, Voet et al. 2002) (figure 3.19).

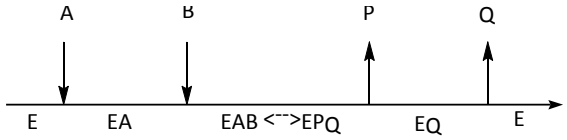


Fig.3.19 Reactants binding and leaving in an ordered sequential bi-bi mechanism

In a **random sequential mechanism**, any substrate can bind first to the enzyme and any product can leave first (Chang 2004, Voet et al. 2002).

3.3.3.2 Ping pong bi-bi (double-displacement)

In the double displacement mechanism, substrate A first binds to the enzyme creating an EA complex, followed by the release of product P leaving a newly formed enzyme complex EF behind (Chang 2004).

Then substrate B will attach to EF, eventually forming product Q and the original enzyme (figure 3.20) (Chang 2004).

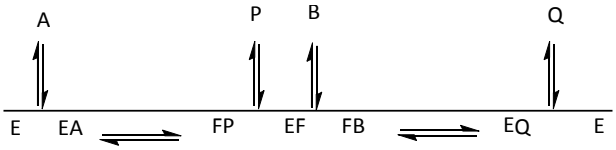


Fig.3.20 Reactants binding and leaving in ping pong bi-bi mechanism

3.4 Enzyme Immobilization

In order for enzymes to be applicable for the industry, methods are necessary to increase their stability and make them reusable (Renneberg 2007, Datta et al. 2013). In certain processes, like in the pharmaceutical or food industry, the enzyme must be fully removed from the system in order to avoid immunoreactions (Renneberg 2007). In addition, a way must be found to separate enzymes from the reaction system (Renneberg 2007). One way to do so is with immobilization (Datta et al. 2013).

Immobilization describes the fixation of an enzyme on a substance, which greatly differs from the substrates and products (Datta et al. 2013). This makes it possible to increase resistance in terms of condition changes such as pH or temperature (Tanaka et al. 1993) and "generate continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity" (Datta et al. 2013, Tanaka et al. 1993)

A large number of immobilization techniques have been developed for continuous processing and reuse (figure 3.21).

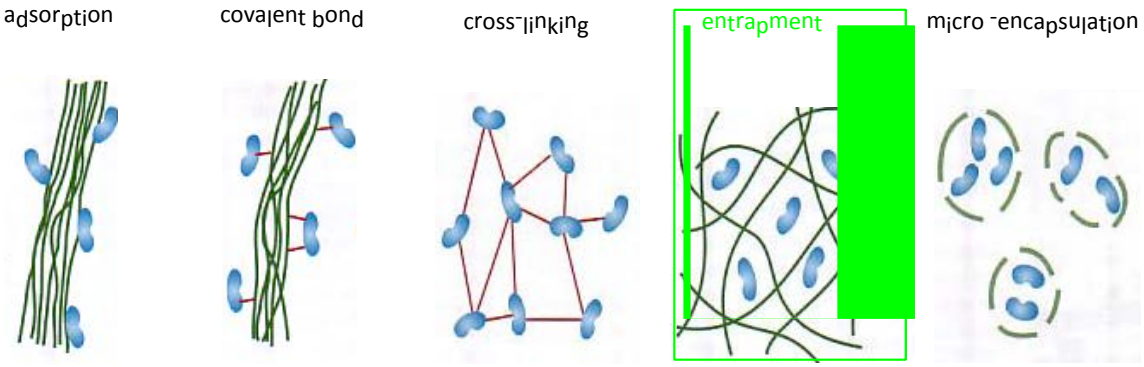


Fig.3.21 Common immobilization techniques (from Renneberg 2007, p.41, box 2.8). Green box marks technique used in this thesis

The type of immobilization must be carefully chosen, in order to avoid a leaching of the enzyme (Gopinath and Sugunan 2004)

3.4.1 Matrix entrapment

The immobilization technique used in this thesis is the entrapment method. By using this method, an enzyme is being included inside a polymer matrix, in which the enzyme is fixed and the substrates are able to diffuse to the enzyme's active site (Sheldon 2007).Advantages are its potential to retain the enzyme activity due to mild reaction conditions and good mass transfer characteristics (Buchholz et al. 2012).

The pores of the network must be bigger than the substrates, so that they are able to reach the enzyme, but at the same time they must be small enough for the enzymes not to leach (Klein et al. 1983, Wang et al. 2004). Using matrices as an immobilization technique always bears the risk of limited diffusion and could lead to substrate depletion and product accumulation at the reaction site, which inhibits the reaction (Guisan 2006).

In this thesis, the components used for immobilization were chitosan and agar. Chitosan is a natural polyaminosaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (figure 3.22) (Krajewska 2004). It is obtained industrially by deacetylation of chitin, a structural component in the exoskeleton of crustaceans (Krajewska 2004, Shahidi and Synowiecki 1991).

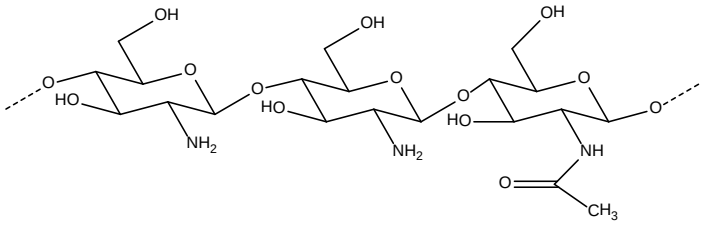


Fig.3.22 Structure of chitosan

Chitosan builds stable polymer networks when mixed with phosphate buffers (Ondruschka 2008). The pKa-value of the amino groups is 6.2, hence giving solubility in acidic solutions at pH smaller 6 (Mi et al. 1999). In this slightly acidic medium the amino groups are positively charged, so that chitosan turns into a polycation which reacts with negatively charged polyanions, e.g.: polyphosphate, resulting in a chitosan-polyphosphate complex to form a gel (figure 3.23) (Mi et al. 1999). Enzymes can be entrapped in such polymer gels, by initially pouring them into the polycation solution (Mi et al. 1999).

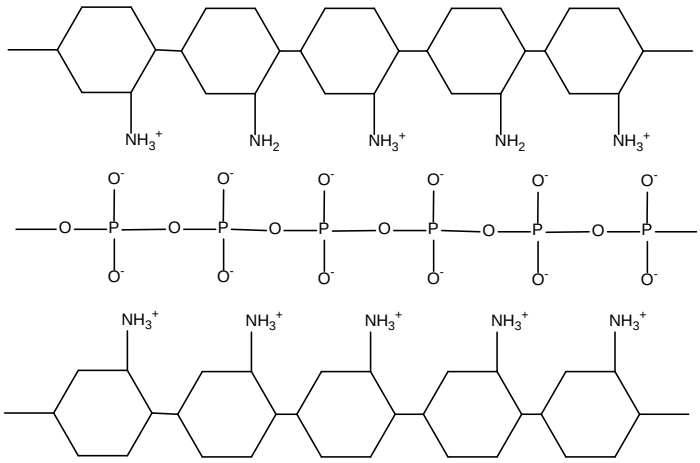


Fig.3.23 Chitosan-PP complex (from Mi et al. 1999)

Agar is a gelatinous polysaccharide which occurs in the cell walls of agarophytes algae (Phillips 2000, www.agargel.com.br). The structure of agar is based upon agarose and agaropectin (www.agargel.com.br). Agarose, representing the fraction which forms the gel and predominant component of agar, is made up of agarobiose units consisting of alternating 3-linked β- D galactopyranosyl and 4-linked 3,6-anhydro-α- L- galactopyranosyl units (figure 3.24) (Praiboon et al. 2006, www.agriorissa.blogspot.de).

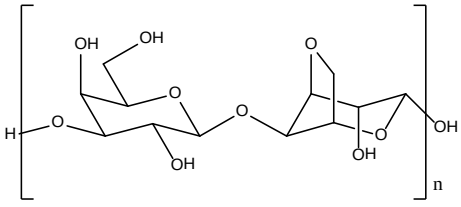


Fig.3.24 Structure of agarose polymer

Agar has the properties of forming a gel when cooled to 32°C-40°C, which will melt at a temperature above 85°C (Koch 1881, www.agargel.com.br). Many reactions carried out using enzymes are performed 40°C, which make agar an interesting solidifying agents, such as gelatin, which melts at this temperature (www.agriorissa.blogspot.de).

Immobilization of enzymes often changes the apparent kinetic parameters, which may be caused by diffusional barriers (Guisan 2006). Diffusion describes the substance transport of a component, which is caused by a gradient of the concentration of the component along the length (Wedler 2004). The substance flux is proportional to the concentration gradient. The proportional constant is called the diffusion coefficient D. The first Fick Law describes the substance transport with regards to its concentration (eq. 3.8) (Fick 1855).

$$\frac{\mathbf{dc}}{\mathbf{dt}} = -\mathbf{D} \cdot \frac{\mathbf{dc}}{\mathbf{dx}} \qquad \textbf{(3.8)}$$

c: concentration [mol/m^3]
t: time [s]
D: diffusion coefficient [m^2/s]

x: position of substance [m]

Knowledge of the diffusion coefficient of the participating components is an important building block for creating an optimal immobilisate. If the substance transport is strongly limited, the efficiency of the catalyst is reduced, since enzymes inside the immobilisate are not supplied with substrates (Buchholz and Kasche 1997). If a slow reaction takes place with a fast substance transport, the substance transport limitation can be neglected. If the reaction is fast and substance transport is limited, a substance transport control is to be expected (Buchholz and Kasche 1997). The mentioned considerations concerning catalyst efficiency after being immobilized can be calculated by the Thiele modulus ϕ (Thiele 1939). With the Thiele modulus it is possible to conclude to what extend a substance transport inhibition must be taken into consideration (Thiele 1939). This is shown by setting the maximum enzymatic reaction rate (v_{max}) in relation with the substance transport and the Michaelis-Menten constant (Buchholz and Kasche 1997). The Thiele Modulus describes how the diffusion influence the activity of the immobilized enzyme (eq. 3.9).

$$\phi = \frac{r_0}{3}\sqrt{\frac{v_{max}}{K_M \cdot D}} \qquad (3.9)$$

ϕ: Thiele modulus [-]
D_{eff}: diffusion coefficient [m^2/s]
r_0 : diffusion distance, pellet radius [m]
v_{max}: maximum reaction rate [mol/m^3s]
K_M: Michaelis constant [mol/m^3]

To quantitatively determine the amount of the limitation, the definition of system effectiveness η becomes necessary (eq. 3.10) (Doran 1995). η describes the relationship between the reaction rate of the immobilized enzyme in the pellet and the reaction rate without substance transport limitation (Doran 1995).

$$\eta = \frac{3}{\phi} \cdot \left(\frac{1}{\tanh\phi} - \frac{1}{\phi}\right) = \frac{k_1}{k_2} \qquad (3.10)$$

η: system effectiveness
ϕ: Thiele modulus [-]
k_1: reaction rate constant of the immobilized enzyme [mol/m^3s]
k_2: reaction rate constant for the solubilized (native) enzyme [mol/m^3s]

The concentration of a solute in the pores is a function of the Thiele-modulus ϕ (eq. 3.8). The system effectiveness η (eq. 3.9) can be described as a function of ϕ for first order reactions (eq. 3.11) (Doran 1995).

$$\eta = \frac{\tanh\phi}{\phi} \qquad (3.11)$$

The effect of the Thiele-modulus ϕ on the system effectiveness can be interpreted by the following terms (table 3.1) (Doran 1995).

Tab. 3.1 Interpretation of the effect of the Thiele modulus on η

Neglectable pore diffusion limitation	$\phi < 0.3$	$\frac{tanh\phi}{\phi} \approx 1$	$\eta \approx 1$
pore diffusion limitation	$\phi > 0.3$	$tanh\phi \approx 1$	$\eta \approx \frac{3}{\phi}$ (sphere)

For small ϕ (<0.3) the effectiveness of the biocatalyst is approximately 1. The concentration of the substrate is not decreasing within the pore and the pore diffusion can therefore be neglected (Buchholz and Kasche 1997). If the value for ϕ is greater than 0.3 the enzyme effectiveness takes value of $\frac{3}{\phi}$ for spherical particles. The reactant concentration tends to reach a zero value towards the interior of the pore. As a result the reaction rate is influenced by the diffusion (Buchholz and Kasche 1997).

3.5 *Euglena gracilis*: the factory for laminaribiose phosphorylase production

Since laminaribiose phosphorylase cannot be obtained commercially, it must be produced by *Euglena gracilis*.

3.5.1 Characterization of *Euglena gracilis*

The *Euglena* family was first discovered in 1830 (Ehrenberg 1830). It is a diverse group containing around 44 genera and 800 species (John et al. 2002). Members of the *Euglena* family are generally wide spread in nature. They exist in free form and colonize fresh water pools and lakes (Hausmann 1985).

E. gracilis is an eukaryotic organism, which has photosynthesizing chloroplasts, enabling growth by autotrophy (Hickmann et al. 2008). In addition, it is also able to nourish heterotrophic (Takenaka et al. 1995). When living heterotrophic, the algae surround the organic material and consume it by phagocytosis (Maruyama et al. 2011).

Eulgena gracilis contain a red eyespot, which enables it to perform phototaxis (Hausmann 1985).Phototaxis describes the movement of organismus towards light sources (www.spektrum.de). In positive phototaxis, the organism moves towards higher light intensities(www.spektrum.de). The mechanism of phototaxis for the algae is performed by the red eyesport partially filtering the incoming sunlight, forcing E gracilis the move towards the light source (Schaechter 2012). Chloroplasts containing pigments of chlorophyll a and b are active when sufficient sunlight is available for a phototrophic growth to produce sugars (Nisbet 1984). Characteristic for *E. gracilis* is the ß-1.3-glucane paramylon, which serves as energy storage for survival in the absence of light (Hausmann 1985).

In general, cell structures of all *Euglenoida* are similar (figure 3.25). They don´t possess a cell wall, but rather a pellicle (Schaechter 2012). The pellicle is a flexible, thin layer protecting the algae and allows it to keep its form (Schaechter 2012).

E. gracilis are generally aerobe, but are also able to tolerate anaerobic conditions, for example in puddles. They have been used as indicators for waste levels in waters (Palmer 1969). Also they need low concentrations of vitamin B12 for growth, which is why they have been initially used in the medical field to determine the amount of B12 in biological solutions (Felski 2004).

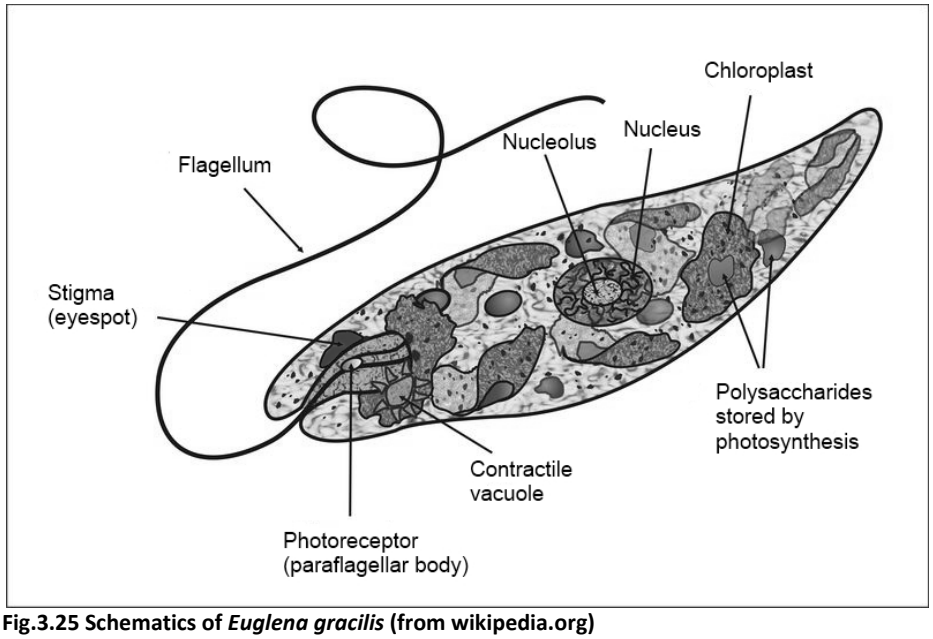


Fig.3.25 Schematics of *Euglena gracilis* (from wikipedia.org)

3.5.2 Growth of microorganisms

For an organism to grow it is depended any physical and nutritional factors (www.amrita.vlab.co.in, Antranikian 2005). pH, temperature, pressure, viscosity, moisture, carbon, nitrogen, sulphur, etc...determine whether or not the cell size and mass of an organism will grow (www.amrita.vlab.co.in).

The growth of *E. gracilis* corresponds to an exponential bacterial growth. It can be modeled in four different phases (figure 3.26) (Zwietering et al. 1990, Fuchs and Schlegel 2007).

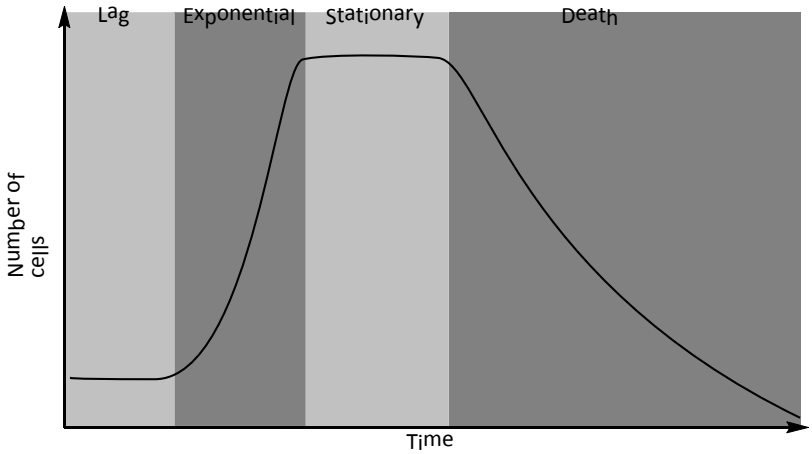


Fig.3.26 Model of the growth of living cells (based on Fuchs and Schlegel 2007)

1. Lag Phase

In the lag phase, the microorganism is placed in the cultivation medium, where it first needs to get adjusted to its new environment (Fuchs and Schlegel 2007). The cellular metabolism takes time to adapt. During this phase the cells will not divide, nonetheless the cell will increases in size (www.generalbacteriology.weebly.com). The length of the lag-phase depends on the condition of the precultures and the new growth conditions (Fuchs and Schlegel 2007).

In case the culture are located in a medium, that has the optimal physical and nutrition factor required, the organism will quickly adapt and start to multiply (Fuchs and Schlegel 2007). The cells, however will take longer to adapt when inserted into a poor medium, that does not have the required factors for growth (Fuchs and Schlegel 2007).

2. Exponential phase

After adaption to the new environment, the cell number increases exponentially as the organisms beginning DNA replication (Fuchs and Schlegel 2007).

3. Stationary phase

The stationary phase will eventually occur, when the nutrient concentration becomes low and the the organism produces toxic metabolites, creating a environment not suitable for growth (Fuchs and Schlegel 2007, www.amrita.vlab.co.in). The cells do not replicate anymore and the growth rate corresponds to the death rate (Fuchs and Schlegel 2007). At this point, the microorganism should be placed into a fresh medium, where it can resume its growth (www.amrita.vlab.co.in).

4. Death phase

When all nutrients are depleted and/or the toxic effect the cells metabolic product cause an autolysis, the number of cells decreases. In this phase, the number death rate is higher than the growth rate (Fuchs and Schlegel 2007).

3.6 Downstream processing

An important field in modern biotechnology is the downstream processing, i.e. the purification of products from fermentation processes (Ladish 2001). This is especially important in the manufacture of pharmaceuticals product such as antibiotics and antibodies, which need to be highly purified before distributed on the market (Renneberg 2007). Downstream processing delivers a purified product suitable for a specific use. For many bioprocesses, up to 80% of the major cost in manufacture is caused by production separation and purification (Schügerl and Hübbuch 2005). Hence it is important to establish downstream processes that reduce overall production costs.

3.6.1 Chromatography

Chromatography describes a separation technique, in which the solution to be analyzed is dissolved in a mobile phase, and is carried through a structure holding material (stationary phase, usually in the form of a column) (http://basicschemistry.blogspot.de). The different properties of the molecules in the mixture cause them to have different interactions with the stationary phase and hence travel at different speeds, and forcing a separation of the molecules (Cammann 2000).

3.6.1.1 Princples of chromatography

The substances eluated from the column create electric signals within a detection system (Kromidas and Kuss 2008). A chromatogram visualizes the signals and will appear as peaks (figure 3.27) (Kromidas and Kuss 2008). Mathematical model parameters are used to describe a chromatographic system and comprehend its changes (Mallik 2008).

The retention time t_R describes the time needed for a substance from sample injection to the detection of a peak maximum the time for a substance (Cammann 2000). Due to characteristic properties, each substance to be analyzed possesses it own specific retention time (Cammann 2000). The flow time of a substance is represented by t_0.

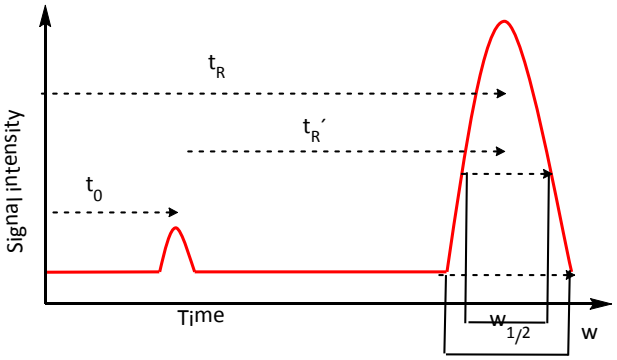


Fig.3.27 Chromatogram and its parameters (based from http://basicschemistry.blogspot.de/)

Substances can only be separated in a chromatographic system if they posses different retention time and therefore specific retention factors *k'*, based on the following equation (3.12) (Cammann 2000).

$$k_A' = \frac{t_R - t_0}{t_0} \qquad (3.12)$$

A values less than one is unfavorable, since the elution rates are too fast for determining retention times (http://basicschemistry.blogspot.de/). Analogously, when k´ is greater than 20, elution rates take long and detection will also be inaccurate. An optimal retention time is between one and five (http://basicschemistry.blogspot.de/).

The selectivity factor is a measure for the separation capability of a chromatographic column (eq. 3.13) (Cammann 2000, http://basicschemistry.blogspot.de/). The greater the difference between the capacity coefficients K, the easier the separation. Optimal values have a range of 1-10.

$$\alpha = \frac{K_2'}{K_1'} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \qquad (3.13)$$

The resolution is a parameter which describes, how great the spaces between the appearing peaks are (Dolan 2002). The resolution is most commonly described by

$$R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} = \frac{1.18(t_{R2} - t_{R1})}{w_{1/2_2} - w_{1/2_1}} \qquad (3.14)$$

In order to obtain a good separation the value of R is ideally 1.5, while 1.75-2.0 represent the acceptable minimum resolution (Dolan 2002).

As seen above, only sharp and symmetrical peaks can deliver optimal separations and hence an efficient chromatographic system (Cammann 2000, http://basicschemistry.blogspot.de/). However, band broadening due to different factor will always occur, and must be limited as best as possible (Cammann 2000, http://basicschemistry.blogspot.de/).

To understand to movement of the analytes within a column, the theoretical plate model should be applied (Kister 1992). By using this model, the substance travels through "successive equilibration chambers, called theoretical plates" (http://www.chem.uoa.gr) (figure 3.28). Within each plate, the

substance to be analyzed completely lies in equilibrium with the stationary and the mobile phase (Kister 1992)

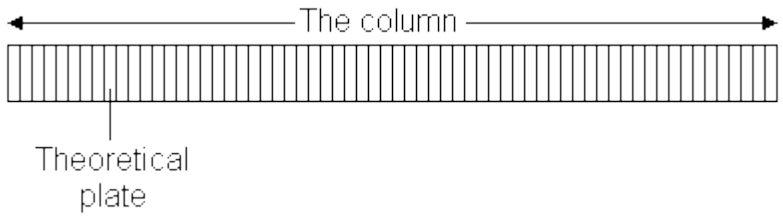


Fig.3.28 Theoretical plate model (from http://basicschemistry.blogspot.de/)

In a more realistic model, the equilibration time is not infinitely fast, as the theoretical plate model suggests, but takes a certain amount of time (Kister 1992, http://basicschemistry.blogspot.de/).The time to equilibrate is depended on factors such as velocity of the mobile phase, kinetics, and column packing (http://www.chem.uoa.gr). The Van-Deemter equation for plate height takes the mentioned factors into account to give a more accurate description of the behavior of an analyte within a column (eq. 3.15) (van Deemter et al. 1956).

$$\mathbf{HETP} = \mathbf{A} + \frac{\mathbf{B}}{\mathbf{u}} + \mathbf{C} \cdot \mathbf{u} \qquad \textbf{(3.15)}$$

HETP: "height equivalent to a theoretical plate", gives information about separation capabilities of a column (Kister 1992).

Eddy diffusion A: takes into the consideration, that the analytes take different routes, with different length, while moving through the column, causing a broadening of the peak. (Otto 2006, van Deemter et al. 1956).

Longitudinal diffusion B: Describes the diffusion of the analyte along the column axis (van Deemter et al. 1956). B is dependent on the viscosity and temperature of the mobile phase (Otto 2006). The analyte will diffuse away from its center of origin to the edges of the column, causing brand broadening (Kister 1992, http://basicschemistry.blogspot.de/).The higher the velocity the more the longitudinal diffusion will decrease (http://basicschemistry.blogspot.de/).

C: This term represents the mass transfer between the stationary and mobile phase, influenced by type of the mobile phase and thickness of the stationary phase (Kister 1992, van Deemter et al. 1956). It is directly proportional to the mobile phase´s velocity. The broadening increases with velocity (http://basicschemistry.blogspot.de/).

u: Describes the velocity of the mobile phase (Kister 1992)

3.6.1.2 Separation techniques in chromatography

One can differentiate between analytical and preparative chromatography (Cammann 2000). In this thesis, thin-layer chromatography (TC), high performance liquid chromatography (HPLC) and high performance anion chromatography (HPAEC) were used.

TC is a physical-chemical chromatographic separation technique (Cammann 2000). Because each compound has a different solubility in a specific medium, and have different affinities towards a stationary phase, each compound will have different travel rates (Cammann 2000). The separation efficiency can be influence by changing the stationary phase or the solvent (Cammann 2000).

HPAEC and HPLC, in the context of this work, are used as **ion-exchange chromatography techniques,** a process in which ions and polar molecules are being separated based on their charge (Weiß 2001). The analyte of a certain charge in the mobile phase will react with oppositely charged molecules of the stationary phase (Weiß 2001, Cammann 2000). The affinity of the analytes in the mobile phase to interact with the stationary column can be influenced pH or ionic strength of the mobile phase (Weiß 2001). Typical ion exchange resins are synthetic polymers or modified dextrane and cellulose materials, typical cationic groups are tertiary or quaternary amines (www.shimadzu.com, www.dionex.com, www.shodex.de).

3.6.1.3 Detection

Detection of the sugar molecules with HPAEC was performed with the Dionex pulsed amperometric detection (PAD) (http://www.dionex.com/en-us/products/ion-chromatography/ic-rfic-solutions/hpae-pad/lp-111613.html). PAD is triple-pulse potential waveform for the detection of carbohydrates in a basic medium (Corradini 2012). On the electrode surface substance encounter specific potentials , resulting in oxidation of analytes (Corradini 2012). In the case of sugar detection, the carbohydrates are oxidized and form a thin oxidation layer on the Ag electrode and the resulting current is measured (Corradini 2012). By reducing the potential again, the oxidation layer is reduced back to the native metal and the electrode surface is renewed (Corradini 2012).

In terms of HPLC analysis, a refractive index detector was used, which is a pure differntial instrument (Titterton and Haney 2008). The detection is performed by measuring changes of the refractive index of eluent passing through the flow-cell (Titterton and Haney 2008). This change will be larger, when the RI difference between sample and mobile phase is larger as well (Titterton and Haney 2008). An RI detector only has narrow applications, since any changes in the eluent composition make a recalibration of the detector necessary (Titterton and Haney 2008).

3.6.2 In-situ product removal (ISPR)

In-situ product removal (ISPR) describes the product separation during the actual reaction process. ISPR systems have the purpose to increase yield, productivity, and high purity of products obtained in a process (Freeman 1993). It is an effective method to obtain the product from thermodynamically unfavorable reactions as well as reduce side reactions and product inhibition (Woodley et al 2008). This is achieved by several effects (Freeman 1993): minimization of interference which allows a continuous reaction at its maximal production level; minimization of product losses resulting from environmental conditions, cross-interactions of product and enzyme or uncontrolled removal from the system; reduction in the number of downstream processing steps. Introducing the ISPR into a process is dependent on many factors, including the mode of operation (batch, continuous, etc.) and specific characteristics of the enzyme (Woodley et al. 2008). However main modes of ISPR systems exist, which are shown in figure 3.29 (Woodley et al. 2008).

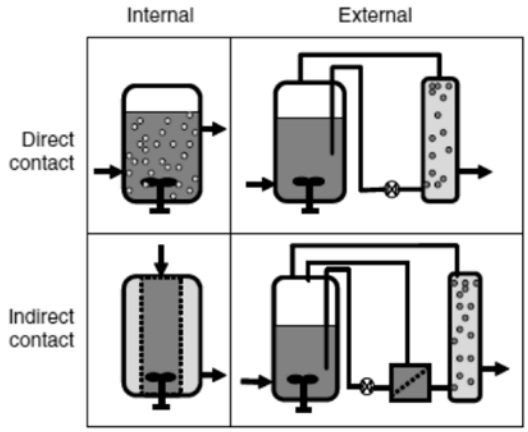


Fig.3.29 Schematics of internal and external ISPR systems (from Woodley et al. 2008)

Relatively few ISPR processes have been established in the industry. The main application is the BIOSTIL® process, which is used for the continuous, fermentative production of ethanol from sugar-containing media.

3.6.2.1 Adsorption of products with zeolites

Adsorption describes the accumulation of substances on the surface of a solid phase (adsorbant) (Bathen und Breitbach 2001). The phenomenon can be used for product separation when different components in a reaction mixture possess different affinities towards the adsorbant (Masel 1996). The pores of the adsorbant can exclude certain molecules based on the particle size (Masel 1996). Due to their good adsorbic properties, zeolites in this study are used as adsorbants to remove the target sugar (laminaribiose) from the reaction mixture by an ISPR process (Fornefett 1999). Zeolites are a wide spread adsorbant in the industry, examples being for the water purification and preparation of

advanced materials (Fornefett 1999). Their biggest use, however, is in the production of laundry detergents (Puppe 1986).

So far more than 200 unique zeolite frameworks have been identified, and over 40 naturally occurring zeolite frameworks are known (International Zeolite Association, Database of Zeolite Structures). Zeolites consist of a microporous primary structure of AlO_4^- and SiO_4 tetraeders in which the aluminum and silicon atoms are bonded to oxygen atoms (figure 3.30) (Cejka et al. 2010). Based on the structural type, a structure of evenly sized pores and/or channels will be build, in which substances can be adsorbed (figure 3.30) (Cejka et al. 2010). The zeolite family can be summarized by the generalized chemical formula (eq. 3.16) (Sherman 1999):

$$M_{X/n}[(AlO_2)_X(SiO_2)_Y]\cdot z(H_2O) \qquad \textbf{(3.16)}$$

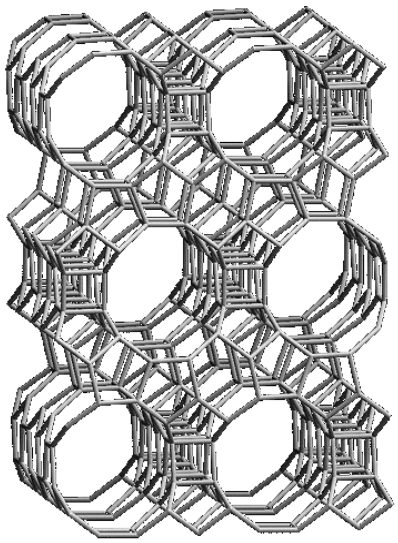


Fig.3.30 Exemplary zeolite structure showing BEA (from International Zeolite Association)

In this thesis, BEA zeolites were used because they are suitable for the adsorption of carbohydrates (Fornefett 1999). The wide pore, high silica zeolite beta was synthesized in 1967 using tetraethyl ammonium hydroxide (Wadlinger et al. 1967). This zeolite is a hybrid of two intergrowing polymorphs termed polymorphs A and B. In both polymorphs, the 12-membered ring pore system is a three dimensional structure, with two straight channels with pore diameters of 0.77 nm x 0.66 nm and 0.56 nm x 0.56 nm (Wadlinger et al. 1967). "Compared with other zeolites, BEA has a high density of stacking defects" (Chaves et al. 2014)

3.6.2.2 Adsorption models

To quantify the amount of substance adsorbed on a surface, different adsorption models can be used (Bathen and Breitbach 2001). Adsorption is usually described through isotherms, that is, the amount of adsorbate on the adsorbant as a function of its concentration at constant temperature (Bathen and Breitbach 2001).

In the simplest case, the adsorbate q is directly proportional to the equilibrium concentration c and put in relation by the **Henry adsorption isotherm**. The proportionality constant H_I corresponds to the Henry constant and as a result Henry´s law is applied (eq. 3.17) (Henry 1803). Equation 3.17 is very useful in terms of screening for adsorbents suitable for an ISPR system.

$$q_i = H_i \cdot c_e \qquad (3.17)$$

The **Langmuir Isotherm** was developed to describe the adsorption on a surfaced with a limited amount of adsorption sites (Langmuir 1918). It is a semi-empirical isotherm with a kinetic and statistical thermodynamic base and is represented by equation 3.18 (Langmuir 1918)

$$\mathbf{q_i = q_{max} \cdot \frac{K_L \cdot c_e}{1 + K_L \cdot c_e}} \qquad (3.18)$$

q_i: fractional coverage of the surface [$g_{adsorbed}/g_{carrier}$]
q_i: maximum concentration of adsorbant adsorbed [mol/l]
c_e: concentration of adsorbant in solution [mol/m^3s]
K_L: Langmuir coefficient [$g_{adsorbed}/g_{carrier}$]

The following assumptions are made in the case of a single adsorbant being adsorbed on the surface of a solid (Masel 1996, Gorecki et al. 1999).

1. "The surface is homogeneous"
2. "All sites are equivalent"
3. "A mono-layer coverage"
4. "There are no interactions between adsorbate molecules on adjacent sites"

The last model which usually describes an adsorption is given by the **Freundlich Isotherm** (Freundlich 1906). "It is an empirical equation presenting the isothermal variation of adsorption of a quantity of gas adsorbed by unit mass of solid adsorbent with pressure" (Vadi and Omidi 2012). In many cases, with increasing amount of adsorbed molecules, the adsorption enthalpy increases as well and the Langmuir isotherm cannot be applied anymore(Kümmel and Worch 1990).Also with increasing covering, the enthalpy decreases logarithmically (Demkov and Navrotsky 2005). The **Freundlich isotherm** describes an adsorption isotherm which, in contrast to the Langmuir isotherm, takes into consideration the adsorption sites at random spreading not being equivalent and that the adsorbed molecules influence each other (Kümmel and Worch 1990). The more particles adsorbed, the more difficult the adsorption of additional molecules becomes (Kümmel and Worch 1990) The Freundlich isotherm is used for multilayer adsorption on irregular surfaces (Liu et al. 2013).

4. Materials and experimental set-up

4.1 Enzymes, chemicals and equipment

4.1.1 Enzymes and Strains

Enzyme	Strain	Characteristics	Source
Sucrose phosphorylase (SP)	*Leuconostoc mesenteroides*		Bitop AG
Glucose isomerase (GI)	*Streptomyces murinus*	fixed within extruded glutaraldehyde cross-linked cells	Novozymes
Laminaribiose phosphorylase (LP)	*Euglena gracilis*	wild type	SAG Göttingen

4.1.2 Chemicals

All chemicals used in this thesis were purchased from Sigma-Aldrich Life Sciences, Merck, Carl Roth, Megazyme, and Fluka.

4.1.3 Equipment

- Autoclave
  - VE-150(Systec)
- Bunsen burner
  - Flame 100 (WLD Tec)
- Centrifuges
  - Avanti J-E w/t rotor JA-10 (Beckman Coulter)
  - Centrifuge 5415 C (Eppendorf)
  - Fresco 21 w/t rotor 75003424 (ThermoScientific-Heraeus)
- Clean bench
  - Lamin Air HLB 2448 (Heraeus)
- Compartment dryer
  - INE 600 (Memert)
- Chromatography refrigerator
  - Unichromat 700 (Uniequip)
- HPAEC
  - Basic device: ICS 3000 (Dionex)
  - Pump: Gradient Pump, Model GPM-2 (Dionex)
  - Pre column: CarboPac PA1, 4*50 mm, (Dionex)
  - Main column: CarboPac PA1, 4*250 mm, (Dionex)
  - Detector: Pulsed Amperometic Detector, model PAD-2 (Dionex)
  - Auto sampler: Series 200 (Perkin-Elmer)
  - Degasser: He 4.6- 5 (pre-pressure 1-2 bar 1.6 mm ID, 3,2 mm OD), EO Regulator Accessory (5-7 Psi) (Dionex)
  - Software: Chromeleon 7.1 (Dionex)
- HPLC
  - Pump: LC-9A, Shimadzu
  - Pre column: SC-G Ca^{2+}, 4,6*10 mm (Shodex)
  - Main column: SC 1011 Ca^{2+}, 300*8 mm (Shodex)
  - Detector: RI Detector, RI 101 (Shodex)

  - Auto sampler: Marathon 816 (Spark Holland)
  - Thermostat: T-6300 Column Thermostat (Merck)
  - Degasser: Degasys DG 1210 (Uniflows)
  - Software: Clarity (DataApex)
- Incubation shaker locker
  - 3033, CTI 5065 (GFL)
  - Titramax 1000 w/t Inkubator 1000 (Heidolph)
- Magnetic stirrer
  - Ret, Rco, Ro (IKAMAG)
- Microwave
  - Space Cube (Philips-Whirlpool)
- Photometer
  - 6315 Photometer (Jenway)
- pH measuring equipment
  - pH meter GMH 3530 (Greisinger Electronic)
  - pH electrode (Greisinger Electronic)
- Pumps
  - IPN (Ismatec)
- Vortexer
  - VV3 (VWR)
  - Vortex Genius 3 (IKA)
- Rotator
- Scales
  - M-Power (Sartorius)
  - R160 P (Sartorius)
- SDS-PAGE
  - Mini-PROTEAN® Tetra System, PowerPac HC (Biorad)
- Ultrasonic devices
  - Sonopuls HD2070, Sonotrode MS72 (Bandelin)
  - Sonorex RK52 (Bandelin)
- Thermal heating block
  - MHR-23 (HLC)
  - HTMR-131 (HLC)
- Water baths
  - 1083 (GFL)
  - Ter2 (IKA)
  - 5 (Julabo)

4.2 Cultivation media

The cultivation media were autoclaved at 121°C and 1 bar for 25 minutes. Temperature sensitive materials were sterile filtrated (0.2 µm pore diameter) and added to the media.

4.2.1 Agar medium for phototrophic cultivation of *E. gracilis*

Tab.4.1 Phototrophic cultivation medium

Substance	Concentration g/l
Peptone	15
Yeast extract	3
NaCl	6
Glucose	1
Agar	12

4.2.2 Kitaoka medium for standard heterotrophic cultivation of *E. gracilis*

Tab.4.2 Kitaoka medium

Substance	Concentration g/l
Peptone	5
Yeast extract	2
Glucose	15
Vitamin B12	10 µg/l
pH	6.8

Preparation of the medium after Kitaoka was realized by separately autoclaving the glucose solution (300 g/l) and peptone-yeast extract solution (Kitaoka et al. 1993). Vitamin B12 (0.5 g/l) was sterile filtrated and stored under absence of light in the refrigerator.

4.3 Strain maintenance

4.3.1 Phototrophic strain maintenance

100 µl of the phototrophic *E. gracilis* from SAG Göttingen were distributed over agar plates (see also 4.2.1). If sealed with parafilm and kept under moderate sunlight sources at room temperature, the cultures are maintainable for many months. Cultures will appear as green colonies after several weeks.

4.3.2 Heterotrophic strain maintenance

The *E. gracilis cells*, with a total cultivation volume of 100 ml. were kept in a 250 ml Erlenmeyer flask, placed in a darkened rotary shaker at 120 rpm (GFL CTI 5065) and cultivated at 29°C. 100 µl of phototrophic strain cells from SAG Göttingen were mixed with 95 ml peptone-yeast extract solution, 5 ml glucose solution (300 g/l) and 2 µl vitamin B12 solution (0.5 g/l). The steps were carried out under

sterile conditions. The adaption to heterotrophic feed takes approximately one week in which the chlorophyll of the chloroplasts is degraded.

Strain maintenance was performed with nearly the same conditions as mentioned above, except 2 ml of inoculums were used, and inoculated every 3-4 days. If experiments with LP were not necessary, *E. gracilis* cultures can be inoculated every 3 weeks, as during growth phase LP concentrations are especially high.

4.3.3 Examination of contaminated heterotrophic strain maintenance

100 µl of *E. gracilis* were distributed over agar plates. If sealed with parafilm and kept in a darkened rotary shaker (GFL CTI 5065) at 37°C for 3 days, cells of *E. gracilis* are nearly invisible after 3 days, showing only slightly green cultures. However at the same elapsed time, if the cultures are contaminated with bacteria they will clearly be visible on the plate.

4.4 Optimization of heterotrophic *E. gracilis* cultivation

4.4.1 pH of the cultivation medium

The Kitaoka medium has a pH of 6.8. To study the effect of the pH on concentrations of the biomass, the pH of the medium was varied, using a citric acid phosphate buffer.

Tab.4.3 used buffer after McIlvaine

pH of buffer solution	0.2 M Na_2HPO_4	0.1 M citric acid	pH in culture medium
	ml	ml	10% buffer volume: 90 % Kitaoka medium
2.6	2.18	17.82	4.1
3.0	4.11	15.89	4.4
4.0	7.71	12.29	5.0

In addition, it was determined whether the growth of the organism is influenced by varying the amount of the buffer in the medium. For this purpose 4 mM Na_2HPO_4 : 8 mM citric acid (10% buffer volume); 16 mM Na_2HPO_4 : 2 mM citric acid (20% buffer volume); 16 mM Na_2HPO_4 : 32 mM citric acid (40% buffer volume) at pH 3.0 was added to the medium.

4.4.2 Temperature of the cultivation

Studies of the phototrophic cultivation of *E. gracilis* indicate a temperature optimum between 25°C-33°C (Kitaya et al. 2005). Therefore cultivations were carried out at 25, 29, and 33°C.

4.4.3 Influence of Vitamin B12 and B1

Vitamin B12 concentrations of 10, 50 and 100 µg/l were added to demonstrate whether or not changes in growth and protein amount occur.

Furthermore, 0.3 mg/l vitamin B1 was added to clarify if it was only needed for a phototrophic cultivation, to reach higher dry masses and protein concentration (Cramer and Myers 1952).

4.5 Cell disintegration

The cell suspension was centrifuged for 10 minutes at 4°C and 3000 rpm (Beckman Coulter Avanti J-E w/t rotor JA-10).

The supernatant was used for the pH and protein mass determination. The pellet was washed with MilliQ H_2O and centrifuged as mentioned above. This step was done twice. The pellet was resuspended in MilliQ H_2O, with a final volume matching 1/20 of the cultivation volume. 0.5 volumes of TRIS-HCl buffer (pH 7.2) was added to the cell suspension. Freeze-Thaw and ultrasonic disintegration were tested for cell disintegration.

4.5.1 Freeze-Thaw

The cultivated cells were frozen at -32°C and slowly defrosted to room temperature. To determine the optimal cycle number, the protein amount and enzyme activity was measured after each cycle.

4.5.2 Ultrasonic disintegration

Ultrasonic disintegration was studied using a Sonopuls HD 2070 by Bandelin with a Microneedle 72, at 50% amplitude and 72% maximum power. To determine the optimal treatment time, samples were taken after 0, 1, 2, 3, 5, 7, 10, and 15 minutes to measure activity and protein amount.

After disintegration, the homogenate was centrifuged for 15 minutes at 4°C and 3000 rpm (Beckman Coulter Avanti J-E w/t rotor JA-10). The supernatant contained the enzyme. All steps were carried out on ice.

4.6 Purification of laminaribiose phosphorylase with ammonium sulfate precipitation and dialysis

Different saturation levels of ammonium sulfate ($(NH_4)_2SO_4$) solutions were first tested (0%-60%) (table 4.4). To a 0.2 ml sample, the necessary amount of saturated ammonium sulfate solution (660 g/l cooled to 4°C) and Na-phosphate buffer (pH 7) was added. The samples were incubated in an ice bath

for 30 minutes and centrifuged for 15 minutes at 4°C and 13400 rpm (ThermoScientific-Heraeus Fresco 21 w/t rotor 75003424). The supernatant was used to determine protein amount and LP activity.

Tab.4.4 Levels of saturation of ammonium sulfate

Saturation	Saturation ammonium sulfate solution	Na-phosphate buffer	Sample with protein
%	ml	ml	ml
0	0	1.8	0.2
20	0.4	1.4	0.2
30	0.6	1.2	0.2
40	0.8	1.0	0.2
50	1.0	0.8	0.2
60	1.2	0.6	0.2

When using large sample volumes, after the proper precipitation level for LP to precipitate has been found, finely dispersed $(NH_4)_2SO_4$ was gently added to the enzyme suspension, until the desired saturation level was reached, in which unwanted proteins precipitate. The suspension was stirred for 30 min at 4°C and afterwards centrifuged for 15 min at 13400 rpm and 4°C (ThermoScientific-Heraeus Fresco 21 w/t rotor 75003424). The supernatant containing the LP was mixed with finely dispersed $(NH_4)_2SO_4$ until the precipitation level in which LP precipitates was reached. The suspension was stirred for 30 min at 4°C and afterwards centrifuged for 15 min at 13400 rpm and 4°C (ThermoScientific-Heraeus Fresco 21 w/t rotor 75003424). The pellet, containing the enzyme was resuspended in 0.1 M sodium phosphate buffer (pH 7.0, 10% of initial enzyme suspension volume). All steps were carried out at 4°C and in an ice bath.

Afterwards dialysis (dialysis tubing, Serva Visking, 27/32, 21 mm) over night against sodium 0.1 M phosphate buffer (pH 7.0) was carried out.

4.7 Characterization of cell growth

To determine the growth rate of *E. gracilis*, samples under sterile conditions (Heraeus Lamin Air HLB 2448) were taken every 24 h.

4.8 Determination of cell dry mass and optical density

A cultivation sample of 2 ml was centrifuged for 10 min at 13400 rpm (ThermoScientific-Heraeus Fresco 21 w/t rotor 75003424). The pellet was resuspended with another 2 ml of the same cultivation sample and centrifuged as mentioned above. The pellet was washed with 1 ml MilliQ H_2O. After another centrifugation step, the pellet was kept in a heating oven (Memert INE 600) at 70°C for 24 h and afterwards the cell mass was determined.

The optical density of the cell suspension was measured photometrically at a wavelength of 578 nm (Jenway 6315 Spectrophotometer).

4.9 Protein analysis after Bradford

The success of the cell disintegration and ammonium sulfate precipitation was investigated with the amount of protein released. For this purpose, samples were quantified using the Bradford assay (Bradford 1976).

4.9.1 Solutions for Bradford assay

Bradford main solution

Tab.4.5 Bradford main solution

Coomassie Brilliant Blue G-250	300 mg
Methanol	300 ml
Ortho-phosphoric acid	600 ml

- Dissolve Coomassie in methanol, and then add ortho-phosphoric acid.

Bradford-reagent solution

Tab.4.6 Bradford-reagent solution

Bradford main solution	150 ml
MilliQ H_2O	850 ml

- After mixture, filtrate coarsely twice, finely ones

4.9.2 Analysis of proteins with Bradford assay

For the measurement of the protein amount, 100 µl samples (diluted if necessary) were added to 5 ml of Bradford-reagent solution and incubated for 15 minutes at room temperature. The extinction was measured at 595 nm (Jenway 6315 Spectrophotometer) against a negative sample with 100 µl MilliQ H_2O. For the correlation of the absorption with the protein concentration, a calibration range with bovine serum albumin (BSA) (0-0.5 g/l) was created.

4.10 Protein analysis with SDS-PAGE

4.10.1 Solutions for SDS-PAGE

10% separation gel (for 2 gels, format 7*10cm from BioRad)

Tab.4.7 10% separation gel

MilliQ H_2O	4.85 ml
30% acryl amide	3.15 ml
1.5 M TRIS-HCl buffer(pH 8.8)	1.25 ml
10% SDS	50 µl
10% APS (ammonium persulfate)	100 µl
TEMED	5 µl

5% stacking gel

Tab.4.8 5% separation gel

MilliQ H_2O	2.85 ml
30% acryl amide	0.85 ml
1.5 M TRIS-HCl buffer(pH 8.8)	1.25 ml
10% SDS	50 µl
10% APS (ammonium persulfate)	25 µl
TEMED	5 µl

Running buffer (pH 8.3)

Tab.4.9 5% running buffer

TRIS base	25 mM
Glycin	250 mM
SDS	0.1 %
Storage	4°C

Laemmli buffer

Tab.4.10 5% running buffer

TRIS-HCl (pH 6.8)	312 mM
Glycerin	5 ml
10% SDS	3.6 ml
ß-mercaptoethanol	1.5 ml
Bromphenolblue	0.02 %

Coloring solution

Tab.4.11 Coloring solution

Coomassie brilliant blue	0.625 g
Methanol	125 ml
MilliQ H_2O	100ml
Acetic acid	25 ml

Discoloring solution

Tab.4.12 Discoloring solution

MilliQ H_2O	80 ml
Methanol	20 ml
Acetic acid	20ml

Protein Standard

Precision Plus Protein Unstained Standard from Biorad has been used (catalogue #161-0363) as a protein size standard for SDS-PAGE.

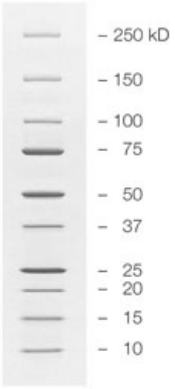


Fig.4.1 Precision Plus Protein Unstained Standard

4.10.2 Analysis of proteins with SDS-PAGE

For the denaturation of the proteins, 15 µl of samples (diluted if necessary) were mixed with 15 µl Laemmli buffer (diluted 1:1) and heat denaturized for 5 minutes. As a control, 15 µl of Precision Plus Protein Unstained Standard were mixed with 15 µl Laemmli buffer (diluted 1:1) and heat denaturized for 5 minutes.

20 µl -25 µl samples were being placed into the stacking gel and the SDS-PAGE run using the following conditions (Biorad Mini-PROTEAN® Tetra System, PowerPac HC).

Tab.4.13 SDS-PAGE program set-up

Program	Time [min]	Voltage [V]	Current [mA]	Output [W]
S1	35	80	30	3
S2	90	120	40	5
S3	90	120	50	10

The separation gel was placed in the coloring solution and shaken for 30 minutes at room temperature. The coloring solution was removed and the gel washed with MilliQ H_2O. The gel was put into the discoloring solution and incubated 30 min. This step was repeated twice and finally kept in the discoloring solution over night. Documentation of the separated proteins was accomplished by photography of the gel.

4.11 Determination of laminaribiose phosphorylase activity

Reactions were carried out in 20 mM glucose, 20 mM G1P and 100 µl enzyme suspensions with final reaction volumes of 2 ml at 30°C for 4 hours in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with thin layer chromatography (TC) and HPAEC.

4.12 Immobilization of sucrose phosphorylase, glucose isomerase, and laminaribiose phosphorylase

4.12.1 Matrix entrapment with chitosan beads

For the immobilization of the enzymes using chitosan, preliminary steps were necessary. 1%-2% chitosan was dissolved in acetic acid (1.0%-2.0% v/v). The chitosan-acetic acid solution was kindly supplied by Waluga (Waluga, Dissertation 2013). The enzyme was then added to the solution. In order to create a bead matrix, the chitosan-acetic acid-enzyme mixture was dropped into stirred non-toxic multivalent counter ions, specifically 10%-15% Na-polyphosphate (pH 5.5, adjusted with either HCl or NaOH). The formed beads within the polyphosphate solution were stirred for an additional 30-120 minutes. The beads were stored for 12 h and then investigated.

Figure 4.2 shows the apparatus used to create the chitosan beads. To find optimal immobilization conditions and receive relatively round and stable beads, the experimental set-ups were varied, such as varying the solution volumes and concentrations, pressures applied, stirring speed, dropping height, flask size, etc. Afterwards the required enzyme was immobilized and tested on its activity. Kinetic experiments of the immobilized enzymes followed when a remaining activity was confirmed.

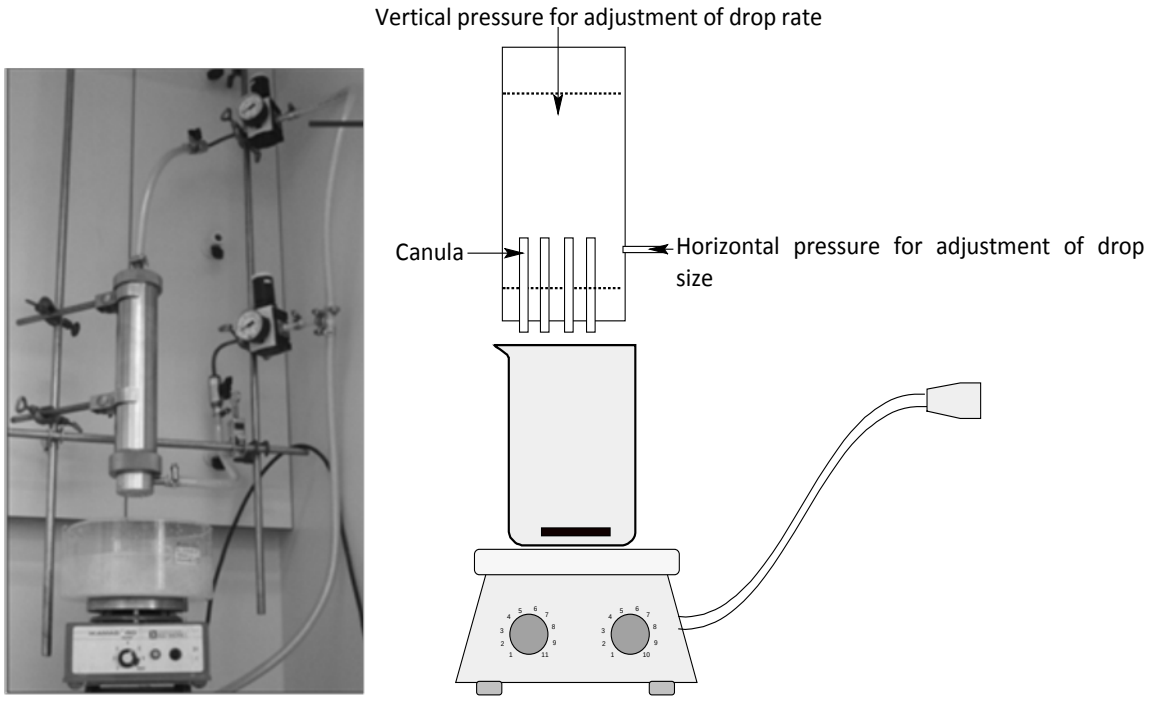


Fig.4.2 Scheme of immobilization apparatus

4.12.2 Entrapment in agar

4.12.2.1 Preliminary experiments with agar

To determine which agar concentrations and experimental set-ups are most suitable for the immobilization of the enzymes, different agar cubes were prepared.

Tab.4.14 Preparation of agar solutions

Agar concentration	1.5%	4%	6%	8%	10%
Agar [g]	0.15	0.4	0.6	0.8	1.0
MilliQ H_2O [ml]	10	10	10	10	10

The agar mixtures were placed in the microwave to obtain a homogeneous solution. Agar begins to solidify at 42°C. The agar was placed in a refrigerator for 30-90 minutes. After the agar was hardened, it was cut in evenly sized cubes and placed in 2 ml Eppendorf cups filled with Sorensen buffer (pH 6.2, 0.5 M). The cups were placed in a rotator, a stirring thermal heating block (HLC MHR-23), an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000), and another non-stirring-heating block (HLC HTMR-131). The conditions were set to 45°C. As a control, the same methods were carried out with agar cubes, not placed in buffer.

4.12.2.2 Enzyme immobilization with agar

The desired agar mixture was prepared as mentioned above (table 4.14). The agar solution was cooled to 45°C, by placing it in a compartment dryer (Memert INE 600, 75°C). The enzymes were then quickly poured and mixed into the liquid agar solution and placed in the refrigerator, for 30-120 minutes, to harden out. The solidified agar mixtures were then cut into even cubes and activity tests carried out. Kinetic experiments of the immobilized enzymes followed when a remaining activity was confirmed.

4.13 Characterization of laminaribiose phosphorylase

4.13.1 Analysis of accumulated LP solution

As the LP solution was obtained by cultivation of *E. gracilis*, preliminary analysis of the solution was required. This included protein and activity determination after Bradford, SDS-PAGE, ammonium sulfate precipitation, and determination of LP activity (see also 4.6, 4.9-4.11).

4.13.2 Effect of temperature on the activity of LP

The effect of the temperature on LP was studied from 35°C-60°C. Reactions were carried out with 200 mM G1P and 200 mM Glc solution with final reactions volumes of 1.5 ml for 6 hours in a thermal heating block (HLC MHR-23). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.13.3 Influence of glucose and glucose-1-phosphate on LP kinetic

The effect of influence of glucose and glucose-1-phosphate on the subsequent reaction during the laminaribiose production was studied by varying glucose concentrations (table 4.15, LP Glc1-6). Analogously, the effect of glucose-1-phosphate on the laminaribiose formation was investigated (table 4.15, LP G1P1-4). Reactions were carried out at 45°C in Sorensen buffer (pH 6.2, 0.5 M) for 6 hours in a thermal heating block (HLC MHR-23) at 900 rpm. 20 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.15 Reaction mixtures for glucose and glucose-1-phopshate influence on laminaribiose formation

No.	G1P mM	Glc mM	LP µl	V_{total} ml	No.	G1P mM	Glc mM	LP µl	V_{total} ml
LP Glc1	20	20	200	1	LP G1P1	20	20	200	1
LP Glc2	20	40	200	1	LP G1P2	40	20	200	1
LP Glc3	20	60	200	1	LP G1P3	60	20	200	1
LP Glc4	20	100	200	1	LP G1P4	80	20	200	1
LP Glc5	20	150	200	1					
LP Glc6	20	250	200	1					

It is of interest to find the best combination of glucose and glucose-1-phosphate to obtain high concentrations of laminaribiose, avoiding the formation of higher oligomers. Therefore it is useful to analyze both substrates in combination, instead being analyzed independently. Reactions were carried out in the same manner as mentioned above.

Tab.4.16 Reaction mixtures for G1P/Glc influence on laminaribiose formation

No.	G1P mM	Glc mM	Ratio Glc/G1P
LPcomb1	20	80	0.25
LPcomb2	20	60	0.33
LPcomb3	20	40	0.5
LPcomb4	20	20	1
LPcomb5	40	20	2
LPcomb6	60	20	3
LPcomb7	100	20	5
LPcomb8	150	20	8
LPcomb9	200	20	10

4.13.4 Kinetic experiments with native LP

For the determination of K_M and v_{Max}, concentrations of glucose-1-phosphate und glucose were varied. Reactions were carried out at 45°C in MilliQ H_2O for 4 hours in a thermal heating block (HLC MHR-23). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.17 Reaction mixtures for LP kinetics, separately varying G1P and Glc

No.	G1P mM	Glc mM	LP µl	V_{total} ml	No.	G1P mM	Glc mM	LP µl	V_{total} ml
natLPG1P A	0.5	200	450	1.5	natLPGlc A	25	5	450	1.5
natLPG1P B	1.3	200	450	1.5	natLPGlc B	25	13	450	1.5
natLPG1P C	3	200	450	1.5	natLPGlc C	25	30	450	1.5
natLPG1P D	8	200	450	1.5	natLPGlc D	25	80	450	1.5
natLPG1P E	20	200	450	1.5	natLPGlc E	25	180	450	1.5

4.13.5 Kinetic experiments with LP immobilized in 1.5% chitosan, 10-15% PP solution

LP was immobilized in 1.5% chitosan and 15% PP solution as described in 4.12.1. Additionally LP was immobilized in 1.5% chitosan and 10% PP solution (see also 4.12.1). Reactions were carried out analogously to the 4.13.4, with the use of a shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000).

Tab.4.18 Reaction mixtures for immob. LP kinetics, separately varying G1P and Glc

%Chi-%PP 1.5-10	%Chi-%PP 1.5-15	G1P mM	Glc mM	Immob. LP µl	V_{total} ml	%Chi-%PP 1.5-10	%Chi-%PP 1.5-15	G1P mM	Glc mM	Immob. LP µl	V_{total} ml
LPA1G1P	LPB1G1P	0.5	200	450	3	LPA1Glc	LPB1Glc	50	5	450	3
LPA2G1P	LPB2G1P	1.3	200	450	3	LPA2Glc	LPB2Glc	50	13	450	3
LPA3G1P	LPB3G1P	3	200	450	3	LPA3Glc	LPB3Glc	50	30	450	3
LPA4G1P	LPB4G1P	8	200	450	3	LPA4Glc	LPB4Glc	50	80	450	3
LPA5G1P	LPB5G1P	20	200	450	3	LPA5Glc	LPB5Glc	50	180	450	3

4.13.6 Kinetic experiments with LP immobilized in agar

LP was immobilized in 4% agar as described in 4.12.2.2. For the determination of K_M and v_{Max} values, concentrations of glucose-1-phosphate were varied. Reactions were carried out at 45°C in 0.5 M Sorensen buffer (pH 6.2) for 2 hours in an incubation locker (Heidolph Titramax 1000 w/t Inkubator 1000). 100 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.19 Reaction mixtures for immob. agar LP kinetics, varying G1P

No.	G1P mM	Glc mM	LP µl	V_{total} ml
Agar LP1G1P	8	200	450	3
Agar LP2G1P	20	200	450	3
Agar LP3G1P	40	200	450	3
Agar LP4G1P	60	200	450	3
Agar LP5G1P	80	200	450	3

4.14 Characterization of sucrose phosphorylase

4.14.1 Analysis of SP solution

SP was kindly provided by Bitop AG. As a preliminary step, the solution was analyzed in terms of activity and protein amount. Therefore protein analysis after Bradford was carried out (see also 4.9). To determine the activity, reactions were carried out in 0.5 M Sorensen buffer (pH 7.0) using 0.1 M sucrose with final reactions volumes of 1 ml for 30 minutes in a thermal heating block (HLC MHR-23). The reactions were initiated by adding 20 µl SP solution. 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.14.2 Effect of temperature on the activity of native SP

The effect of the temperature on SP was studied from 30°C-50°C. Reactions were carried out in 0.5 M Sorensen buffer (pH 7.0) and 300 mM sucrose with final reaction volumes of 1.5 ml for 2 hours in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.14.3 Kinetic experiments with native SP

For the determination of K_M and v_{Max}, concentrations of sucrose were varied. Reactions were carried analogously to 4.14.2 at 45°C. 20 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.20 Reaction mixtures for SP kinetics

No.	Sucrose mM	SP µl	V_{total} ml
SP A	10	20	1.5
SP B	50	20	1.5
SP C	100	20	1.5
SP D	150	20	1.5
SP E	200	20	1.5
SP F	300	20	1.5

4.14.4 Kinetic experiments with SP immobilized in chitosan and agar

SP was immobilized in 1.5% or 2% chitosan and 10% or 15% PP solution as described in 4.12.1 and in 4% agar as described in 4.12.2.2. For the determination of K_M and v_{Max} values of immobilized SP concentrations of sucrose were varied. Reactions were analogously to 4.14.2 at 45°C in 0.5 M Sorensen buffer (pH 7.0 for Chitosan immob. SP, pH 6.2 for agar immob. SP). 100 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.21 Reaction mixtures for immob. SP kinetics

%Chi-%PP			Sucrose	Immob. SP	V_{total}	4% Agar
1.5-10	1.5-15	2-15				
			mM	µl	ml	
A1	B1	C1	0.75	20	3	Agar1
A2	B2	C2	2	20	3	Agar2
A3	B3	C3	4.6	20	3	Agar3
A4	B4	C4	12	20	3	Agar4
A5	B5	C5	28	20	3	Agar5
A6	B6	C6	100	20	3	Agar6
A7	B7	C7	200	20	3	

4.15 Characterization of glucose isomerase

4.15.1 Kinetic experiments with GI granules/suspension

For the determination of K_M and v_{Max}, concentrations of fructose were varied. Reactions were carried out at 45°C in MilliQ H_2O for 30 minutes (2 hours for GI suspension) in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with HPLC.

Tab.4.22 Reaction mixtures for GI kinetics

No.	Fructose	GI granules	V_{total}	No.	Fructose	GI suspension	V_{total}
	M	g/l	ml		M	µl	ml
GI A	0.03	0.10	1.5	natGI A	0.06	190	1.5
GI B	0.06	0.10	1.5	natGI B	0.17	190	1.5
GI C	0.11	0.10	1.5	natGI C	0.38	190	1.5
GI D	0.22	0.10	1.5	natGI D	1.00	190	1.5
GI E	0.40	0.10	1.5	natGI E	2.25	190	1.5
GI F	0.56	0.10	1.5				

4.15.2 Kinetic experiments with GI granules/suspension immobilized in chitosan and agar

GI granules/suspension was immobilized in 1.5% or 2% chitosan and 10-15% PP solution as described in 4.12.1 and in 6% agar as described in 4.12.2.2. For the determination of K_M and v_{Max} values, concentrations of fructose were varied. Reactions and sample analysis were carried out analogously to 4.15.1.

Tab.4.23 Reaction mixtures for immob. GI kinetics

%Chi-%PP			Fru	GI	V_{total}	6 % Agar	%Chi-%PP			Fru	GI susp.	V_{total}	6 % Agar
1.5-10	1.5-15	2-15					1.5-10	1.5-15	2-15				
			M	mg	ml					M	µl	ml	
GI A1	GI B1	GI C1	0.06	0.10	3	Agar GI1	susGI A1	susGI B1	susGI C1	0.06	190	3	Agar susGI1
GIA2	GI B2	GI C2	0.17	0.10	3	Agar GI2	susGIA2	susGI B2	susGI C2	0.17	190	3	Agar susGI2
GI A3	GI B3	GI C3	0.38	0.10	3	Agar GI3	susGI A3	susGI B3	susGI C3	0.38	190	3	Agar susGI3
GI A4	GI B4	GI C4	1.00	0.10	3	Agar GI4	susGI A4	susGI B4	susGI C4	1.00	190	3	Agar susGI4
GI A5	GI B5	GI C5	2.25	0.10	3	Agar GI5	susGI A5	susGI B5	susGI C5	2.25	190	3	Agar susGI5

4.16 Bienzymatic reaction system

4.16.1 Effect of temperature on native bienzymatic reaction

The effect of the temperature on a bienzymatic system with SP and GI was studied from 30°C-50°C. Reactions were carried out in 0.6 M Sorensen buffer (pH 7.0) and 300 mM sucrose with final reaction volumes of 3 ml for 2 hours in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.16.2 Kinetics of native bienzymatic reaction

Substrate concentrations of 300 mM, 500 mM, and 800 mM were used. Reactions and sample analysis were carried out analogously to 4.16.1.

4.17 Trienzymatic reaction system

The bienzymatic system was broadened to the trienzymatic reaction by adding LP. The reaction was carried out in 0.6 M Sorensen buffer (pH 7.0) with a final reaction volume of 5 ml for 6 hours at 38°C in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.17.1 Effect of temperature on native trienzymatic reaction

The effect of the temperature on the trienzymatic system was studied from 35°C-60°C in a thermal heating block (HLC MHR-23). Reactions and sample analysis were carried out analogously to 4.17.

4.17.2 Kinetics native trienzymatic reaction

The effect of the substrate (sucrose) on a trienzymatic system was studied. Substrate concentrations of 200 mM and 600 mM were used. Reactions were carried out analogously to 4.17.1 at 38°C. 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.17.3 Influence of pH on native trienzymatic reaction

The influence of the pH on the reaction was investigated in the range of pH 6.2-pH 8.5 at 45°C for 6 hours. Reactions were carried out in Sorensen buffer (with adjusted pH) and 500 mM sucrose with final reaction volumes of 1.5 ml in a thermal heating block (HLC MHR-23). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.17.4 Immobilized trienzymatic system in a batch reactor

The enzymes were immobilized in chitosan and agar as described in 4.12. Reactions were carried out in Sorensen buffer (with adjusted pH) and 500 mM sucrose at 45°C with final reaction volumes of 40 ml with 0.6 mM $MgSO_4$ as GI activator in an incubation shaker locker (Heidolph Titramax 1000 w/t Inkubator 1000). 20 µl samples were taken at defined time periods and analyzed with HPAEC.

4.18 Modeling of reaction systems

The reactions were modeled using a combination of a Runge-Kutta-Integration and a Simplex-Algorithm for the iterative parameter optimization with the ModelMaker3® software (Cherwell Scientific Publishing Ltd.). Besides product formation, phosphorolysis, hydrolysis, subsequent reactions, equilibrium, and inhibitions must be taken into consideration.

4.19 Analysis and purification of the carbohydrate products

4.19.1 Thin layer chromatography

4.19.1.1 Solutions for thin layer chromatography

Mobile Phase

Tab.4.24 Mixture for mobile phase

Substance	Water	acetic acid ethyl ester	isopropyl
Ratio [%]	12	3	1

Color solution

Tab.4.25 Mixture for color solution

5 g/l Potassium permanganate ($KMnO_4$)	5 g
1 M NaOH	250 ml

- Storage under absence of light

4.19.1.2 Qualitative analysis with thin layer chromatography

For qualitative analysis of the sugar mixtures thin layer chromatography was carried out to quickly determine the production of laminaribiose and higher laminaroligomers. Samples of 0.5 µl were spotted on the lower end of a silica gel plate (Polygram S,L G/UV_{254}, Machery-Nagel GmbH&Co. KG/Düren). The plate was dried and placed for 15-20 minutes in a thin layer chromatography chamber, containing the mobile phase. The plate was placed in the color solution for 20 seconds, washed with water and carefully dried. $KMnO_4$ reacts with reducing carbohydrates, where darkened spots will appear on the now violet plates.

4.19.2 Analytical HPLC/HPAEC

For a quantitative analysis of samples, HPLC/HPAEC systems were used. Samples were heated at 95°C for 5 minutes to denaturate the enzyme (HLC MHR-23) and centrifuged to obtain a homogeneous sample (Eppendorf Centrifuge 5415 C).

4.19.2.1 Mobile and stationary phase

For HPAEC, an anion exchange column with an attached pre column (column: Dionex CarboPac PA1, 4*250 mm; pre column: Dionex CarboPac PA1, 4*50 mm) was used with 0.1 M NaOH as the main mobile phase. Samples were eluted at 25°C and a flow rate of 1 ml·min^{-1} within a helium-degassed system. Detection was carried out with an amperometric pulsed detector with a gold electrode (Dionex PAD-2). Sample volumes of 10 µl were eluted with the following gradient:

Tab.4.26 HPAEC gradient programming

Time	Eluent A (main eluent)	Eluent B
	0.1 M 50%-NaOH	1 M NaAc in 0.1 M 50%-NaOH
min	%	%
0 – 6.3	100	0
6.3 - 15	80	20
15 - 35	100	0

For the quantification of the standard solutions (glucose, fructose, sucrose, laminaribiose, laminaritriose, laminaritetraose, glucose-1-phosphate, laminaripentaose), concentrations between 0.05 g/l < c < 0.1 g/l were used.

The HPLC system quantified solutions which only contained glucose, sucrose and fructose. A Shodex column and pre column (column: SC 1011 Ca^{2+}, 300*8 mm; pre column: SC-G Ca^{2+}, 4.6*10 mm) was used with a column temperature of 80°C, with MilliQ H_2O as an isocratic eluent. Samples were eluted at a flow rate of 1 ml·min^{-1} with measuring times of 20 minutes. The calibration area for the carbohydrate standard was between 1 g/l < c < 8 g/l.

4.19.2.2 Detection

Figure 4.3 shows a representative chromatogram (raw data extracted from Dionex Chromeleon 7.0, visualized with Sigma Plot 11.0) with the carbohydrates expected to be used and produced in this thesis.

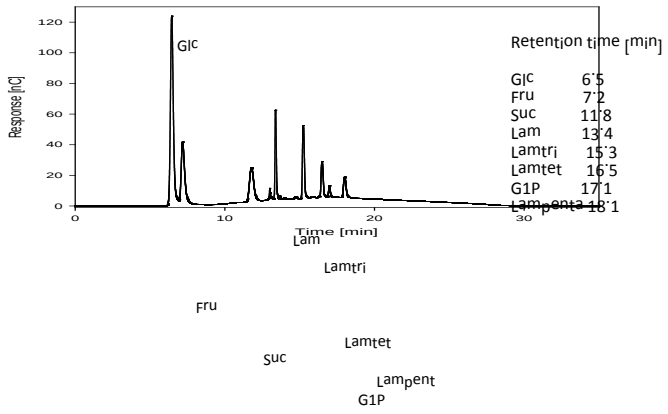


Fig.4.3 Chromatogram after HPAEC analysis of 0.05 g/l standard carbohydrates solution

4.19.3 Reaction integrated product adsorption with zeolites

Different BEA zeolites as adsorbants were investigated and tested. This part is mainly based on the work and results in the PhD thesis 2013 of Waluga (Waluga, Dissertation 2013). Detailed experimental set-ups and results can also be found there.

Analysis of adsorption experiments was realized by measuring the supernatants with HPAEC.

4.20 ISPR for the production and purification of laminaribiose

4.20.1 ISPR system with different zeolites types

The effect of product adsorption by using different zeolite types was investigated. The reactions were carried out with final reaction volumes of 5 ml for several days at 45°C using an ISPR system (see figure 3.29). 80 µl samples were taken at defined time periods and analyzed with HPAEC.

Tab.4.27 Reaction mixtures for internal direct contact ISPR

	R1 native	R2 BEA50 powder	R3 BEA150 powder	R4 BEA150 extrudate
0.7 M sucrose	1 g	1 g	1 g	1 g
Sorensen (pH 6.2, 0.5 M)	5 ml	5 ml	5 ml	5 ml
SP (1/8 trienzym. system)	8.3 µl	8.3 µl	8.3 µl	8.3 µl
LP (1/8 of trienzym. system)	190 µl	190 µl	190 µl	190 µl
GI (1/4 trienzym. system)	166 mg	166 mg	166 mg	166 mg
200 g/l zeolite		1 g	1 g	1 g

5. Results and Discussion

The aim of this thesis was to develop a trienzymatic system to produce laminaribiose from sucrose using sucrose phosphorylase, glucose isomerase, and laminaribiose phosphorylase. This will be studied as a model for multienzymatic reactions.

5.1 Improvement of production of laminaribiose phosphorylase from *Euglena gracilis*

5.1.1 Maintenance of the *Euglena gracilis* strains

The strain maintenance of E. gracilis must be realized either by continuous propagation onto a fresh medium or a phototrophic maintenance on nutrient agar. Freezing, as done for many bacteria, is not possible.

The effect of the inoculation source was studied. A phototrophic culture on an agar plate was used as an initial inoculum for a switch into a heterotrophic cultivation in absence of light. A phototrophic culture needs approximately one week until its metabolism has been switched to heterotrophic cultivation. Afterwards it was immediately used for the propagation of a culture.

Results indicate that the preparation of a new culture from a phototrophic culture from agar plates or from the algae collection Göttingen is possible without loss of dry mass, protein amount and enzyme activity (table 5.1).

Tab.5.1 Acquired cell dry masses, protein amounts, and enzyme activities with different heritage of strain cultures: continual = continuous strain culture; agar = strain culture from agar plate; algae collection = strain culture from suspension of algae collection from Göttingen (cultivation at 29°C, 120 rpm)

	Strain culture	Cell dry mass [g/l]	Protein amount [g/l]	LP suspension activity [U/l]
pH 4.1	Continual	2.71	0.48	22.92
	Agar	2.70	0.45	25.59
	Algae collection	3.19	0.42	25.70
pH 6.8	Continual	0.66	0.19	3.50
	Agar	0.83	0.18	4.44
	Algae collection*	0.23	0.01	0.00

*disturbed during growth (pH at end: 7.7)

After switching to heterotrophic cultivation, similar characteristics were detected. The failed cultivation at pH 6.8 from the phototrophic culture of the algae collection from Göttingen was caused by the unexplainable rise of the pH to 7.7.

Strains cultures could be kept alive without contamination and mutations for several weeks by frequent propagation on agar. A short-termed “reactivation” of a phototrophic culture from an agar plate or algae collection for the heterotrophic production of laminaribiose phosphorylase was also

possible without any difficulties. The cells lost their chlorophyll after three days and the culture could be used as an inoculum after one week.

5.1.2 Optimization of *Euglena gracilis* heterotrophic growth

The objective of the optimization of *E. gracilis* cultivation is to increase the biomass and especially the LP content and activity.

5.1.2.1 Influence of pH on *E. gracilis* growth

The pH is an important factor influencing the growth (Santek et al. 2009). The effect of pH was investigated in terms of the growth patterns by carrying out several cultivations in buffered (pH 4.1) and unbuffered (pH 6.8) medium at 29°C and 120 rpm (figure 5.1).

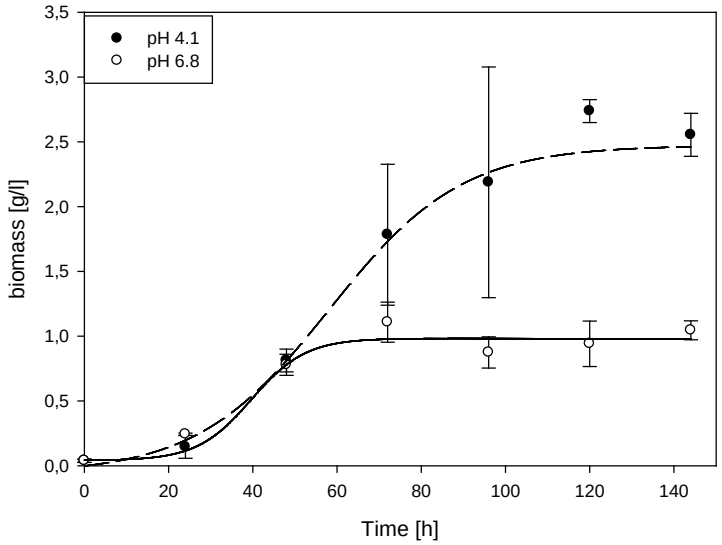


Fig.5.1 Influence of pH on cell growth at pH 4.1 and 6.8 within 144 h of cultivation

The cultivation curves in both media show a 24 h lag-phase, with a beginning exponential growth between 24-30 h. However at pH 6.8 the cell growth stops after 48 h, while the exponential phase for pH 4.1 lasts until 120 h. Afterwards the stationary phase for pH 4.1 is reached with a 150% higher biomass concentration in pH 4.1 than in pH 6.8.

With citric acid-phosphate buffer, the optimum was found at a pH of 4.1, resulting in a 65% increase of cell biomass compared with the unbuffered medium at pH 6.8 as used by Kitaoka (figure 5.2).

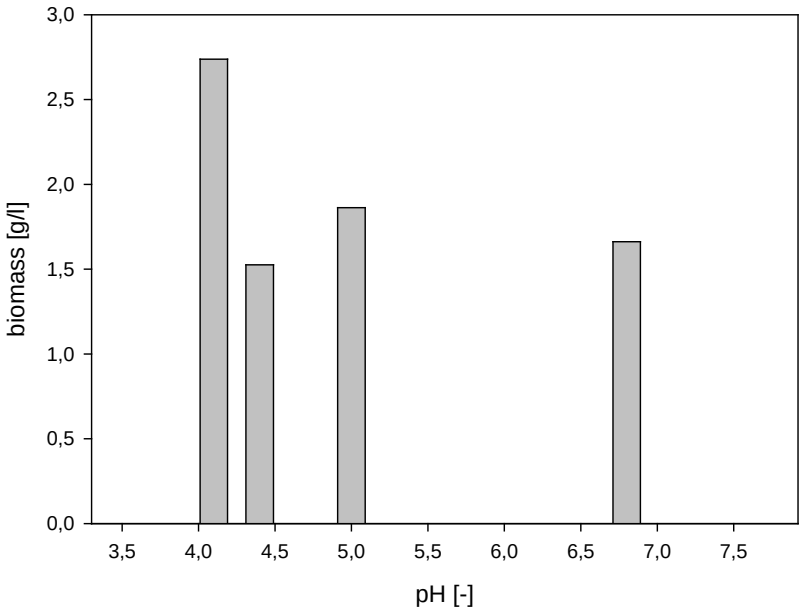


Fig.5.2 Cell bio mass after 144 h of cultivation (25°C, 120 rpm) at pH 4.1, 4.4, 5.0 adjusted with citric acid-phosphate buffer and the unbuffered Kitaoka medium at pH 6.8 (see also table 4.3 for exact compositions of the buffers used)

In addition, it was determined whether the growth of the organism is influenced by varying the amount of the buffer in the medium (figure 5.3). The buffers added had the following compositions; 4 mM Na_2HPO_4 : 8 mM citric acid (10% buffer volume); 16 mM Na_2HPO_4 : 2 mM citric acid (20% buffer volume); 16 mM Na_2HPO_4 : 32 mM citric acid (40% buffer volume). It turned out that the capacity of the buffer does not influence the biomass production. Instead it depends on the pH of the buffer (figure 5.3).

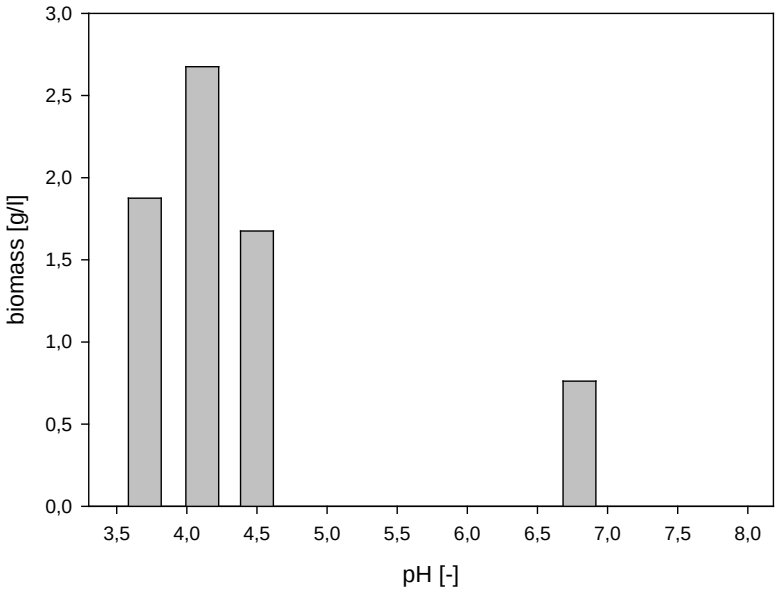


Fig.5.3 Cell dry mass after 144 h of cultivation (25°C, 120 rpm): pH 3.7 (40%), 4.1 (20%), and 4.5 (10%), 0.1 M citric acid-phosphate buffer and the unbuffered Kitaoka medium at pH 6.8

Highest biomass yields were reached at pH 4.1 (20% buffer volume) with 2.7 g/l. Using pH 3.7 (40% buffer volume) and 4.5 (10% buffer volume) yields of 1.7 g/l were obtained, corresponding to 47%

decrease when compared with pH 4.1. The lowest concentration was obtained using the unbuffered Kitaoka medium.

The microscopic examination of the cells after phototrophic and heterotrophic cultivation (pH 4.1 and pH 6.8) showed a significant difference in their morphology (figure 5.4).

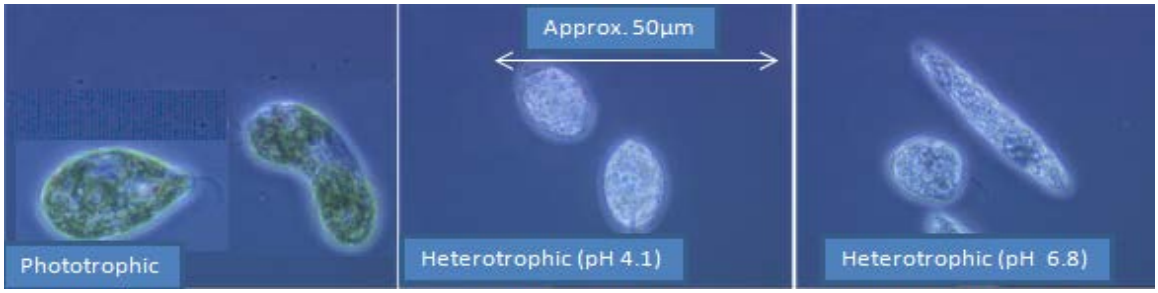


Fig.5.4 Microscopic examination of cell morphology of phototrophic/heterotrophic (pH 4.1, pH 6.8) cultivated cells.

In the phototrophic cultivation the cells appear to have varying form with lengths between 30-60 µm; mainly they seem to be elongated, but can easily switch into a spherical shape. The cell compartments are clearly visible, with the chloroplasts being easily identifiable. The heterotrophic cells appear to take a yellowish color due to the degradation of the chlorophyll. At pH 4.1 the cells have a spherical shape with a diameter of 20 µm and a high mobility. At pH 6.8 the cells are less mobile and have an elongated form. At a phototrophic cultivation the cells must move towards the higher, solar-light richer water layers (phototaxis) (Bhaya 2004). It was observed that even though phototaxis of heterotrophic cells at pH 6.8 was missing, leading to a quick sedimentation, the movement with the flagella was still existent. This was not the case for heterotrophic cells at pH 4.1. It is possible that the missing mobility and variability of the cells at pH 4.1 cause a conservation of the cell´s energy which instead is used for an increased production of cell dry mass.

5.1.2.2 Influence of temperature on *E. gracilis* growth

E. gracilis possesses a large temperature optimum (Kitaya et al. 2005). Therefore the range between 25-37°C was investigated. 29°C is used as a reference as used in Kitaoka´s experiments (Kitaoka et al. 1993). The effect of temperature was investigated in terms of the growth behavior of *E. gracilis* (figure 5.5).

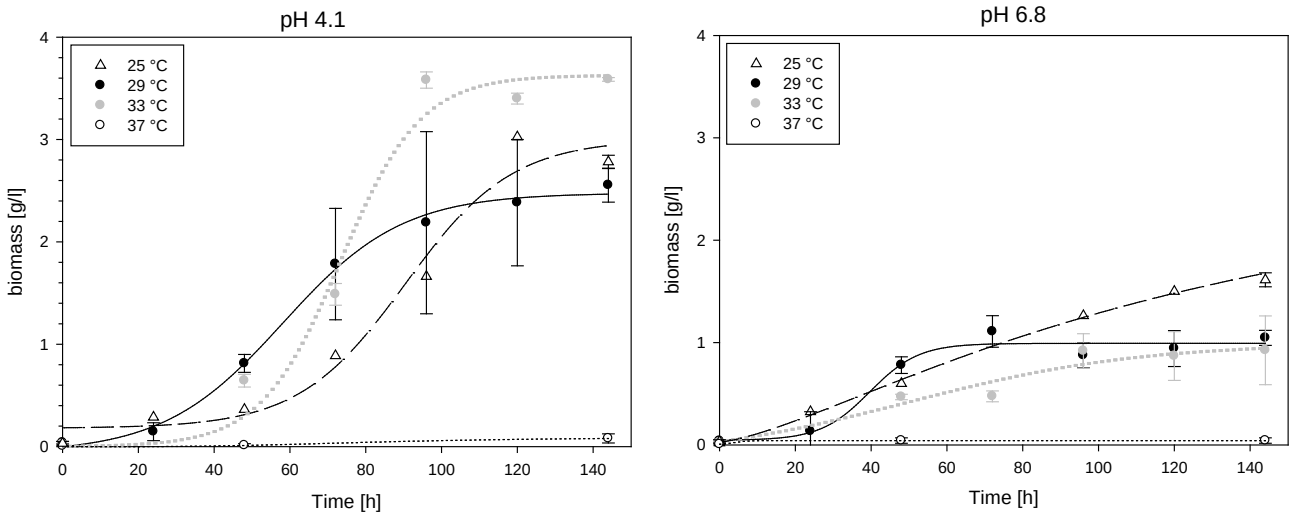


Fig.5.5 Influence of temperature on cell growth at pH 4.1 and 6.8 within 144 h of cultivation

The highest biomass concentrations were obtained for pH 4.1 at 33°C (figure 5.5). This corresponds to an increase for over 100 % in comparison with the biomass concentrations produced at pH 6.8 with a temperature of 25°C. Additionally the exponential phase of pH 4.1 at 33°C is the highest. No growth was observed at 37°C for both pH 4.1 and 6.8.

In comparison with pH 6.8, the lag phase at pH 4.1 is elongated. This is due to precultures, used as inoculums, which were delivered from pH 6.8 in unbuffered medium. As a result, it takes the cells longer to get adjusted to their new environment. At a pH of 6.8, temperatures in the range of 25°C and 33°C seem to force a shortened lag-phase and an extension of the exponential phase, whereas the growth rate is less at 33°C then at 25°C.

Interestingly, the temperature optimum is highly dependent on the pH used (figure 5.6).

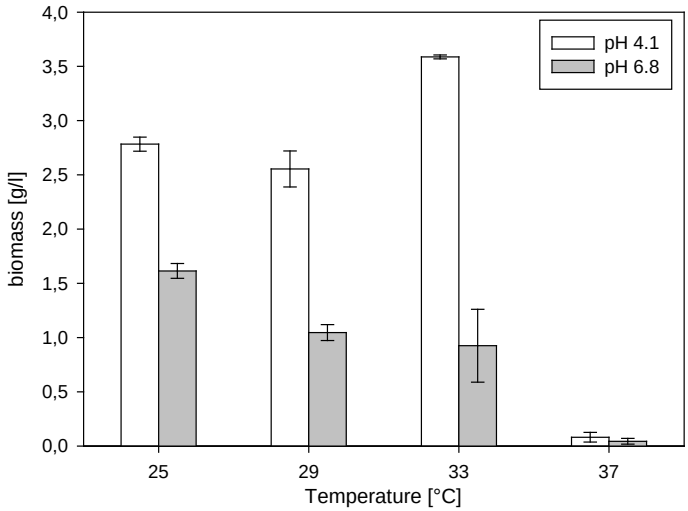


Fig.5.6 Biomass content after 144 h of cultivation for different temperatures at pH 4.1 and 6.8 (120 rpm)

Considering temperature variations at pH 6.8, an increase in temperatures results in the decrease of biomass. The opposite direction was found for pH 4.1, where the optimum biomass growth occurred

at 33°C. No growth was observed at 37°C. The difference in temperature in comparison with variation of pH could be explained by analyzing the morphology of *E. gracilis* (figure 5.4). It can be deduced that low pH triggers stress in the organism, which forces an adapted metabolism and cell structure, resulting in higher cell dry mass yields and making it more resistant to changing environmental conditions, such as higher temperatures (Danilov and Ekelund 2001).

5.1.2.3 Influence of vitamins on *E.* gracilis growth

Yeast extract contains no vitamin B12. Studies indicated that B12 is needed for *E. gracilis* to grow in the absence of light (Felski 2004). As a result, vitamin B12 was added in different concentrations (table 5.2, figure 5.7).

Tab.5.2 Influence of vitamin B12 (10, 50, 100 µg/l) on the cell dry mass production, protein content and enzyme activity after 144 h of cultivation (29°C, 120 rpm)

	pH 4.1			pH 6.8		
Vitamin concentration [µg/l]	10	50	100	10	50	100
Cell dry mass [g/l]	2.81	2.98	2.65	0.53	0.70	0.66
Protein amount [g/l]	0.40	0.39	0.43	0.1	0.12	0.12
Protein ratio [$g_{Protein}/g_{cell\ dry\ mass}$]	0.14	0.13	0.16	0.19	0.17	0.18
Enzyme activity [U/l]	13.49	13.88	14.86	4.86	6.06	3.02
Spec. enzyme activity [U/g protein]	33.73	35.59	34.56	48.60	50.50	25.17

There are no significant notable differences in dry mass, protein amount, and enzyme activity observed in both pH 6.8 and pH 4.1.

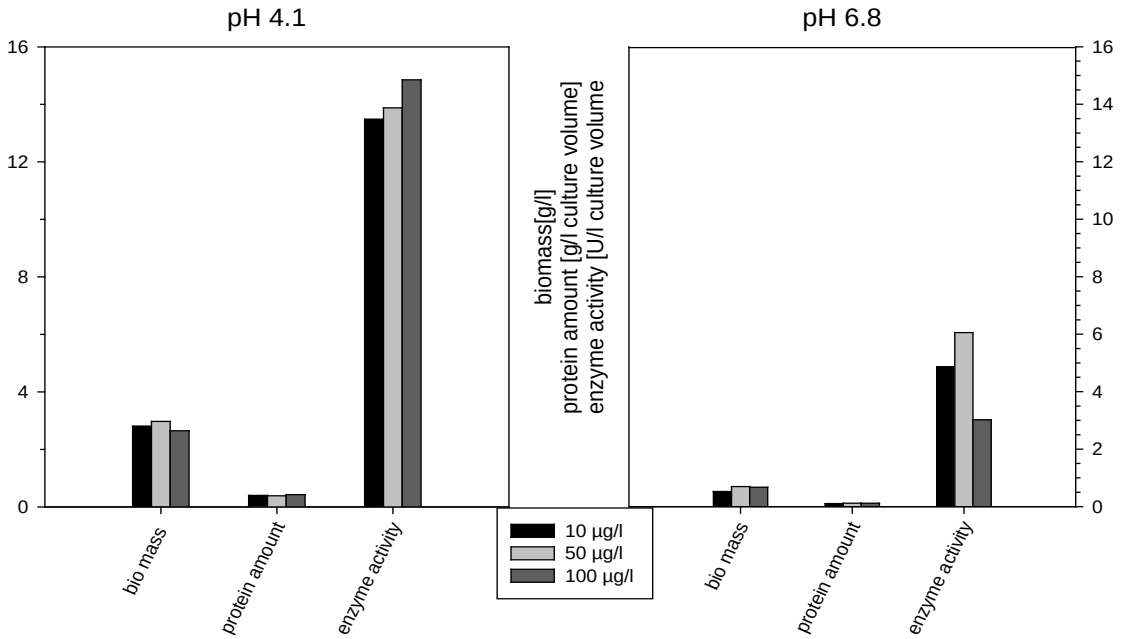


Fig.5.7 Influence of vitamin B12 (10, 50, 100 µg/l) on the cell dry mass production, protein content and enzyme activity after 144 h of cultivation (29°C, 120 rpm)

No biomass growth occurred without adding vitamin B12. Increasing the concentration of vitamin B12 from 10 µg/l to 100 µg/l shows no significant effect on biomass production, protein content and laminaribiose phosphorylase activity in the buffered (pH 4.1) and the unbuffered medium (pH 6.8). Results of pH 4.1 are more homogenous than at pH 6.8. Biomass concentrations are between 2.6 and 2.9 g/l, with protein amounts at 0.4 g/l culture volume. At pH 6.8 biomass concentrations of 0.5 g/l to 0.7 g/l were obtained. The protein amount is at 0.1 g/l. A trendless variation of enzyme activity was observed. *E. gracilis* possesses a ribonucleotide-reductase (E.C. 1.17.4.2) which is responsible for the synthesis of DNA-nucleotides. Ribonucleotide-reductase is dependent on vitamin B12 (Hamilton 1974). So in the absence of vitamin B12 the DNA synthesis is inhibited. This explains the non-existent growth of cultures without adding of vitamin B12 as stated by Cramer and Meyers (Cramer and Meyers 1952). The proposed minimum vitamin B12 concentration of 10 µg/l is sufficient to sustain the ribonucleotide-reductase during culture growth (Kitaoka et al. 1993).

Additionally, the possible effects of vitamin B1 on the cultivation of *E .gracilis* were examined (figure 5.8). Vitamin B1 is an important factor for cell growth, as it is a co-factor for enzymes responsible for cell respiration and energy deliverance (Nakazawa et al. 2003). Even though it is found in yeast extract, it can be destroyed during the autoclaving process of the medium (Beadle et al. 1943). Vitamin B1 is a necessity because *E. gracilis* lacks the synthesis pathway for the pyrimidine ring which contributes to the production of vitamin B1 (Shigeoka et al. 1987).

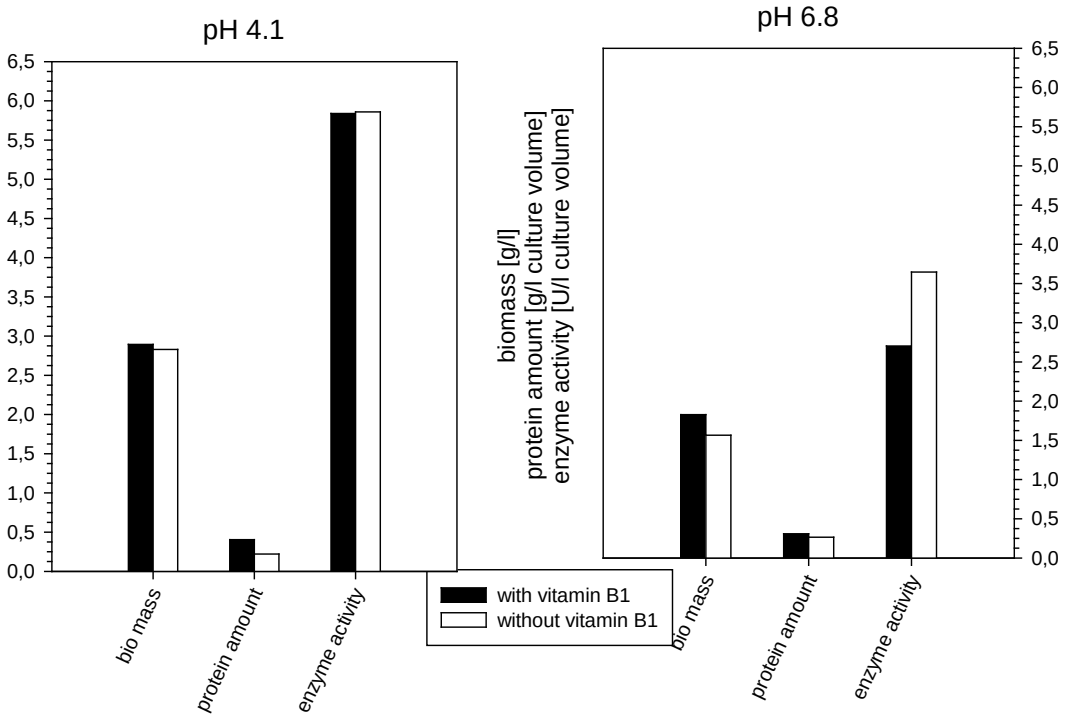


Fig.5.8 Influence of vitamin B1 (0.3 mg/l) on the cell dry mass production, protein content and enzyme activity after 144 h of cultivation (25°C, 120 rpm)

It turned out that there are no significant influences on the cultivation by adding additional vitamin B1 at pH 4.1. Only the cultivation at pH 6.8 showed a noticeable difference in the enzyme activity. Without adding vitamin B1 the enzyme activity was at 3.6 U/l culture volume corresponding to a 35 % rise in comparison with the culture where vitamin B1 was added. It is likely that the cells get enough vitamin B1 from the yeast extract.

5.1.3 Optimization of cell disintegration

The cultivation for the production of LP lasted for six days and then the cells were harvested. Laminaribiose phosphorylase catalyzes the intracellular formation of the storage carbohydrate polymer paramylon (β-D-(1→3) glucane). The enzyme is therefore not secreted. For this reason ultrasonic and freeze-thaw disintegration for cell disruption were studied (figure 5.9). Before starting the disruption experiments, autolysis of cells were tested within the culture medium and after washing twice with water (table 5.3).

Tab.5.3 Investigation of possible autolysis in the supernatant before actual cell disintegration

	Medium	1. Wash	2. Wash	Suspension (as control)
Protein conc. [g/l]	0.018	0.057	0.037	0.146
Protein mass [mg]	2.7	4.28	1.48	3.29

Results show that autolysis is of circumstantial meaning. The low protein concentrations in the supernatants indicate that an autolysis barely takes place after washing with MilliQ H_2O. The protein concentration in the medium is based on the used peptone and probably the autolysis itself, which can occur during the stationary phase. The released protein mass during the washing is probably caused by mechanical stress (vortexing) and the sudden change of environmental conditions.

By confirming the autolysis being negligible, ultrasonic and freeze-thaw disintegration were carried out (figure 5.9). The “zero”-value corresponds to the suspension from the autolysis experiments (table 5.3), scaled to match the culture volume.

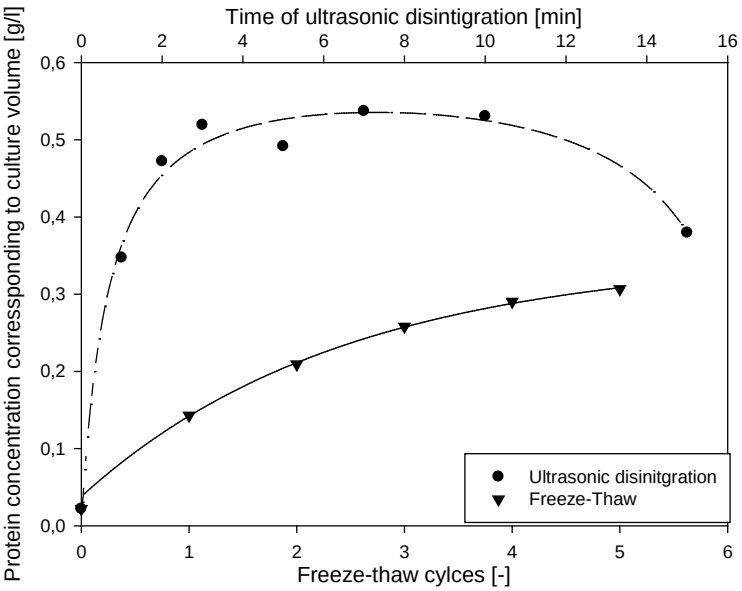


Fig.5.9 Protein (cultivation at pH 6.8, 25°C, 120 rpm) secretion during disintegration via ultrasonic and freeze thaw (frozen to -32°C)

Using ultrasonification, a maximum protein concentration of 0.52 g/l culture volume was reached after three minutes. This corresponds to a protein mass of 53.7 mg in the cell homogenate (15 ml). The rapid decrease after 10 minutes to a value of 0.35 g/l culture volume is due to ultrasonic degradation of protein.

Freeze-thawing gave a maximum protein concentration of 0.3 g/l, thus being 33 % less effective than ultrasonification. Since freeze-thawing also needs much more time for only one cycle, ultrasonification was used for all further studies.

An enzymatic activity reaction of a 5x freeze thawed sample and a 7, 10 and 15 minutes ultrasonification sample were carried out to check on possible damages to laminaribiose phosphorylase (table 5.4).

Tab.5.4 Specific activities after ultrasonic disintegration and 5x freeze-thaw cycles

	Duration	Enzyme activity U/l	Goodness of fit R^2	Specific enzyme activity $U/g_{protein}$
Ultrasonic disintegration	7 min	149.41	0.96	41.74
	10 min	122.68	0.83	34.69
	15 min	80.45	0.85	31.79
Freeze-Thaw	5 cycles	88.31	0.75	43.20

Comparison of the specific activity shows the degrading effect of heat and stress on the enzyme during the ultrasonification. The enzymatic activity decreased by 24 %from 41.7 $U/g_{protein}$ after 7 minutes to 15 minutes treatment, probably due to denaturation of laminaribiose phosphorylase. After the fifth freeze-thaw cycle an enzyme activity of 88.1 U/l was obtained and equaled to a reduction of 40% in comparison with ultrasonic disintegration after 7 minutes. However the specific enzyme activity is

comparable with a value of 43.20 U/$g_{protein}$. Given that disintegration with freeze-thaw is more gentle, since no heat is produced as opposed to ultrasonification, this seems to be the maximum enzyme activity in the cell homogenisate, with regards to protein amount.

5.1.4 Concentration and purification of laminaribiose phosphorylase

After the release of the proteins from the cell, the homogenisate containing laminaribiose phosphorylase must be purifed.

The precipitation was studied, using an ammonium concentration range at which the enzyme precipitates while retaining its activitiy (figure 5.10).

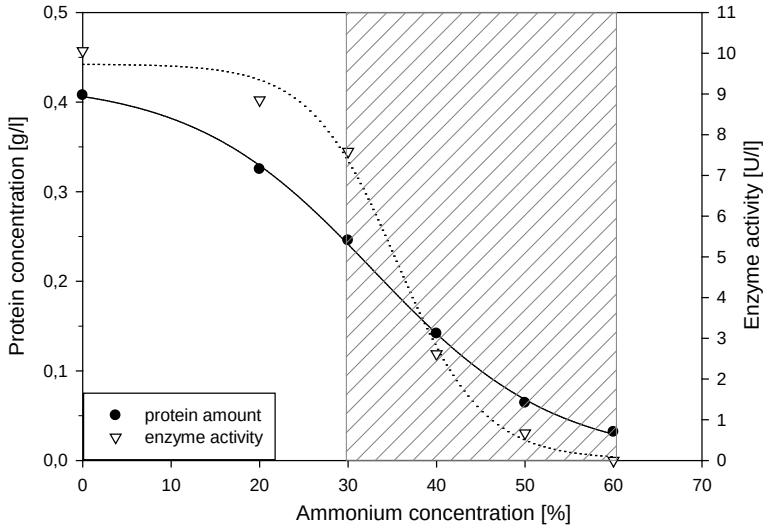


Fig.5.10 Ammonium sulfate precipitation of an enzyme suspension (marked area marks the suitable area as proposed by Goldemberg et al. 1966)

Results show that the enzyme activity, beginning at 30% saturated ammonium sulfate concentration, more strongly decreases with rising ammonium sulfate concentration than the protein concentration (figure 5.10). At 60% there is no activity left. This observation is in line with the data generated by Goldemberg, who used fractions from 33% to 60% for further purification (Goldemberg et al. 1966). Kitaoka only performed a precipitation up to 30 % and separated the proteins in the supernatant by using anion exchange chromatography (Kitaoka et al. 1993). In order to be able to get a purification as well as a concentration of the enzyme in the suspension, Goldembergs attempt was used, where the pellet from the fraction between 30-60% is resuspended in a smaller volume and dialyzed.

After the proper concentration range was found, precipitations using a higher culture volume (800 ml concentrated by 60%) were carried out, followed with dialysis (table 5.5).

Tab.5.5 Protein concentration and enzyme activity before and after ammonium sulfate precipitation and dialysis (800 ml culture volume at pH 4.1, 29°C, 120 rpm

	Cell disintegration homogenate	Suspension after precipitation and dialysis
Protein mass [mg]	214	62
Enzyme activity [U/l]	1482	1611
Spec. enzyme activity [U/g protein]	139	210
Yield of protein mass after precipitation [%]		29
Purification factor		1.5

During cultivation a cell dry mass of 2.4 g/l was reached. After concentrating the suspension to 1/40, ultrasonification was performed. The protein concentration in the homogenate was 10.7 g/l with a specific enzyme activity of 139 U/g protein. After the precipitation and dialysis process, the specific enzyme activity could be increased by 51% to 210 U/g protein. Protein mass was reduced by nearly 70%.

5.1.5 Summary: Optimization of LP production

The composition of the cultivation medium has a great effect on the production of the cell dry mass in *E. gracilis*.

The heterotrophic maintenance can be carried out for a long period (at least 3 months) without the culture being contaminated or changed. Reactivation of a phototrophic culture on agar plates for the use of a heterotrophic cultivation in the dark is possible within a period of time (7 days).

Heterotrophic cultivation was pH and temperature optimized compared to Kitaoka´s method (Kitaoka et al. 1993). It is recommendable to keep the precultures at pH 4.1, 33°C, using citric acid-phosphate buffer (pH 2.6), to reduce the lag phase and hence the cultivation time for the production of laminaribiose phosphorylase. Under these conditions, biomass can be harvested at the beginning of the stationary phase after 96 hours. With respect to vitamin B12, 10 µg/L as suggested in literature is sufficient (Kitaoka et al. 1993). Addition of vitamin B1 for cultivation is not necessary when yeast extract is used as complex nutrient component.

The cell disintegration is best carried out via ultrasonification. Ammonium sulfate precipitation can be applied as a purifying and concentration step. The fraction with laminaribiose phosphorylase is found between 30-60 % saturation.

5.2 Characterization of laminaribiose phosphorylase

5.2.1 Analysis of accumulated LP solution

The purity of the enzyme solution was controlled with SDS-PAGE. It was found that nearly no other proteins occurred in the solution, represented by a hardly visible band at approx. 100 kDA (figure 5.11).

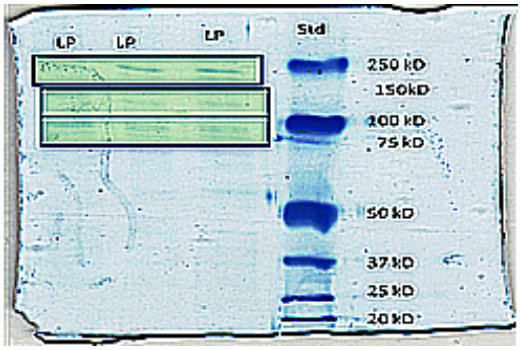


Fig.5.11 SDS-PAGE of the obtained enzyme solution of *E. gracilis* cultivation (for purpose of better visibility, the figure was modified using Adobe Photoshop CS)

Laminaribiose phosphorylase appears as a dimer at approximately 250 kDa and as a monomer at approximately 120 kDa. This is in accordance to the observations found in literature (Kitaoka et al. 1993).

Activity tests confirmed qualitatively and quantitatively, that the enzyme solution containing laminaribiose phosphorylase catalyzes the condensation of glucose-1-phosphate and glucose resulting in the production of laminaribiose. Samples taken after 0, 240, and 1440 min were spotted on thin layer chromatography (figure 5.12a).

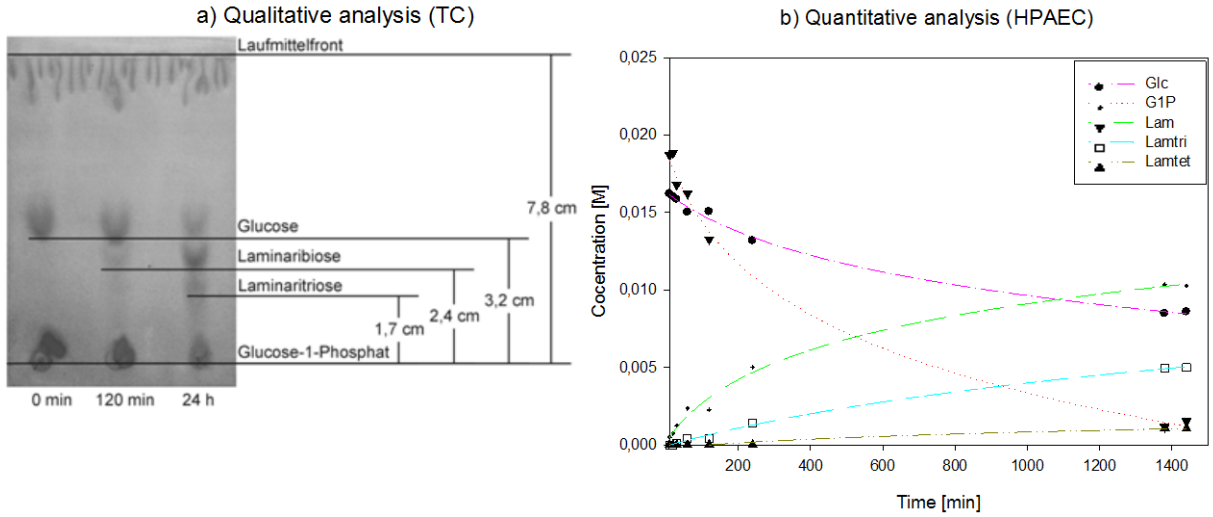


Fig.5.12a/b Analysis of activity test of LP using a) thin layer chromatography and b) HPAEC

The TLC´s show a reduction of substrate concentration while new bands are produced with progressing time. Laminaribiose appears after 120 minutes and after 24 h a second band becomes visible. The

second band is most likely laminaritriose. HPAEC analysis shows a stronger decrease for G1P in comparison to Glc, caused by glucosylation of laminarbiose to laminaritriose (Maréchal 1967), clearly shown in the graph (figure 5.12b). Mass balances of the substances stay relatively constant from 0-240 minutes, which however are decreased by 24% after the 24 h mark. This may be caused by reaction to laminaritetraose, -pentaose, -hexaose and –heptaose, as shown by Goldemberg (1966), which were produced in low yields and therefore not detected by HPAEC. If the reaction is carried out beyond the measured time scale, it is to be assumed that the subsequent reactions to higher oligomers will eventually consume all of the laminaribiose, as after 1400 minutes laminaritriose and laminaritetraose are still showing an increase in their concentration. This is in accordance to the literature (Goldemberg et al. 1966).

5.2.2 Effect of temperature on the activity of LP

The effect of the temperature on LP was studied from 35°C-60°C. The optimal temperature was found between 42°C-45°C (figure 5.13). Literature proposes a temperature optimum of 37°C, which however was not determined by experiments (Goldemberg et al. 1966). 37°C was merely set for the enzymatic assays carried out. Therefore it is likely that the optimum temperature has indeed a higher value than stated in Goldemberg. Temperatures above 60°C may very likely cause a decay of the enzyme.

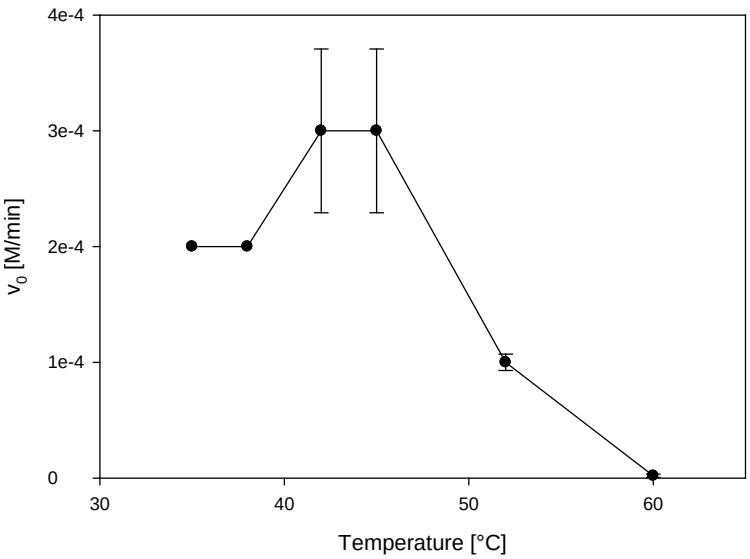


Fig.5.13 Temperature dependence of the initial reaction rates of laminaribiose formation by LP. Initial reaction mixture with 200 mM G1P and Glc

Kitaoka investigated the stability of LP between 35°C, 40°C, and 45°C in Tris-HCl buffer pH 6.4-8.2 (Kitaoka et al. 1993), stating LP being stable at temperature below 45°C, which is in accordance with the results. Temperatures above 45°C cause a denaturation of the enzyme and therefore a rapid decrease of its activity. From 32°C-40°C the enzyme has a reduced activity which rapidly increases beyond 40°C.

5.2.3 Kinetic investigations of LP

Laminaribiose phosphorylase not only produces laminaribiose, but also promotes side reactions, in which laminaribiose consequently reacts with glucose-1-phosphate to produce higher oligomers as laminaritriose and -tetraose (figure 5.12b) (Goldemberg et al. 1966). With regards to laminaribiose and the formation of its higher oligomers, it is useful to investigate the influence of glucose and glucose-1-phosphate on LP kinetics. It can be assumed that a high concentration of glucose will favor the laminaribiose formation reaction, therefore encouraging G1P consumption and reducing the formation of higher oligomers.

5.2.3.1 LP kinetics influenced by glucose

To study the kinetics influenced by glucose, several reactions were carried out by maintaining the concentration of G1P constant and varying the concentrations of glucose. It is obvious that the initial rate increases with rising glucose concentration (figure 5.14).

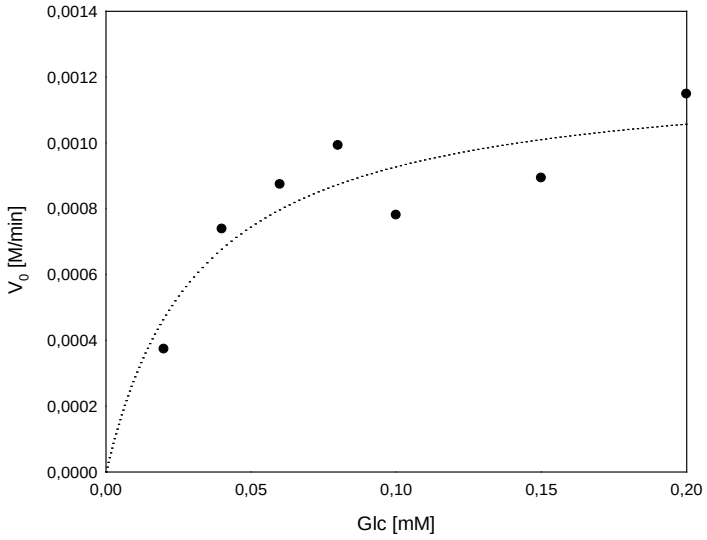


Fig.5.14 Initial reaction rate of Lam formation with varying Glc from 20 mM-200mM. Initial reaction mixture with 25 mM G1P in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

From the plot it can be seen that a full saturation is reached at approximately 200 mM Glucose.

Higher oligomers of laminaritriose and -tetraose were also obtained (figure 5.15a/b). Different effects are observed, namely the formation of laminaribiose from glucose and glucose-1-phosphate, the consumption to laminaritriose from laminaribiose and the consumption of laminaritriose producing laminaritetraose. When the formation rate is equal to the consumption rate, a maximum appears in the graph, until the consumption rate exceeds the formation rate. Laminaritetraose also reaches a maximum and is eventually consumed into laminaripentaose, indicated by the decrease of

laminaritetraose concentrations after 30 minutes. Due to detection limits of sugar analysis laminaripentaose could not be determined.

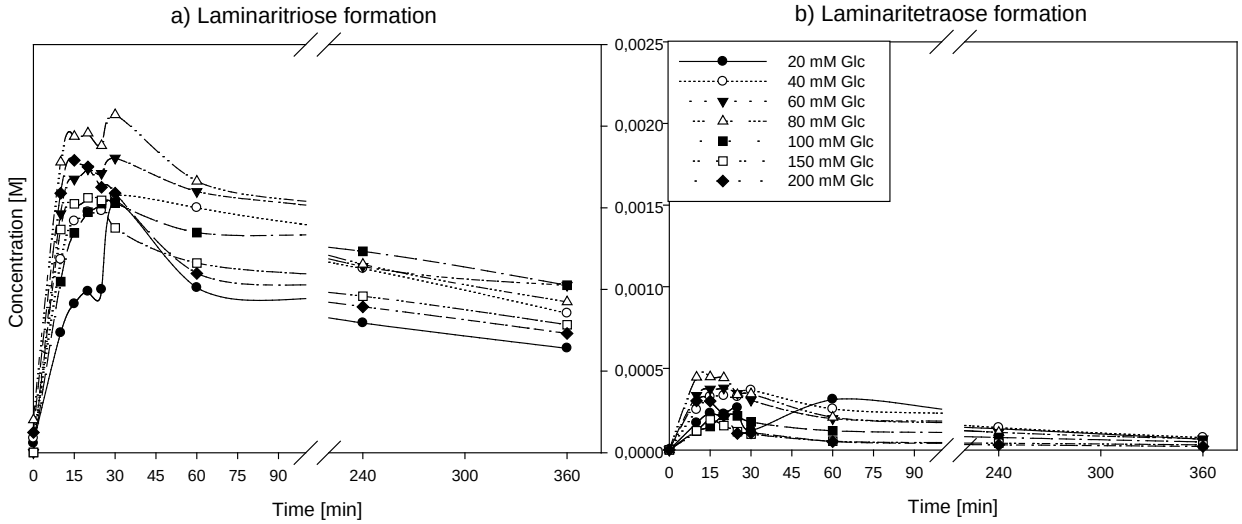


Fig.5.15 Influence of higher oligomers formation with varying Glc from 20 mM-200mM. Initial reaction mixture with 25 mM G1P in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

To get a better insight about the glucose influence on the production of the oligomers and the desired product, it is useful to consider the percentage that each product represents in relation to the total amount of products. Hence it is defined, and analogously is true for the other products, as:

$$\%\mathbf{Laminaribiose} = \frac{[\mathbf{Laminaribiose}]}{[\mathbf{total\ products}]} \cdot \mathbf{100} \qquad \textbf{(5.1)}$$

$$\%\text{Laminaribiose} + \%\text{Laminaritriose} + \%\text{Laminaritetraose} + \%\text{Laminaripentaose} = 100$$

Based on equation 5.1 a vertical bar chart can be created, representing the percentage amount of the products formed (figure. 5.16), with mass balances calculated for 6 hours reaction time. For a better overview, exemplary charts of 20 mM, 100 mM, and 200 mM are shown. As can be seen from the graphs, the percentages of higher oligomers tend to decrease with increasing glucose concentrations. So higher glucose concentrations indeed contribute to reducing the formation of the subsequent products. A high concentration of glucose would tend to shift the ratio of products towards laminaribiose. As a result, less G1P would be shared to react and produce higher oligomers. The assumption can be made, that the grade of formation of polymerization of higher oligomers is reduced, when increasing the glucose concentration.

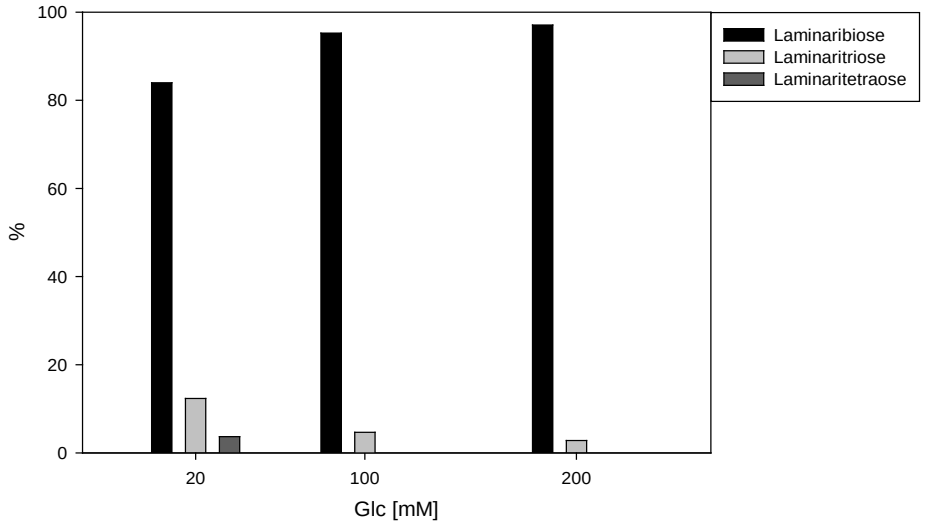


Fig.5.16 Percentage values of products obtained after 6 hour reaction with varying Glc concentrations. Initial reaction mixture with 25 mM G1P in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

5.2.3.2 LP kinetics influenced by glucose-1-phosphate

Analogous to the influence of glucose, the influence of G1P on laminaribiose production was studied (figure 5.17). It can be seen that in general the laminaribiose obtained increases with increase of the of glucose-1-phosphate concentration. However, only minor differences between the reaction rates for 60 mM and 80 mM are observed. This would suggest that the full saturation is reached at approximately at 70 mM G1P. It is possible that the ratio of Pi to G1P mainly sets the equilibrium point of the reaction steps, as suggested by Kitaoka (Kitaoka et al. 1991).

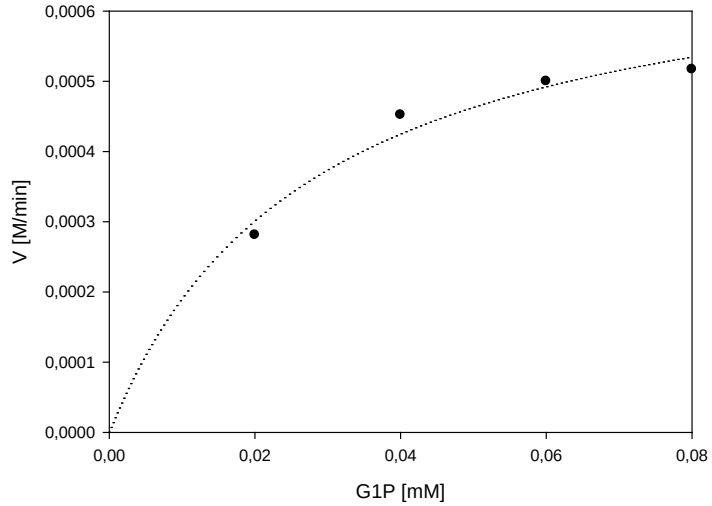


Fig.5.17 Influence of Lam formation with varying G1P from 20 mM-80 mM. Initial reaction mixture with 25 mM Glc in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

Higher oligomer concentrations are obtained with higher concentrations of glucose-1-phosphate (figure 5.18). For a concentration of 20 mM G1P, the laminaribiose represents 87% of the products,

while laminaritriose and laminaritetraose represent 12% and 1% respectively after 6 hours reaction time. When 80 mM were used, the product percentages shifted in favor of the higher oligomers with 25% laminaritriose, 22% laminaritetraose, 9% laminaripentaose and only 44% laminaribiose.

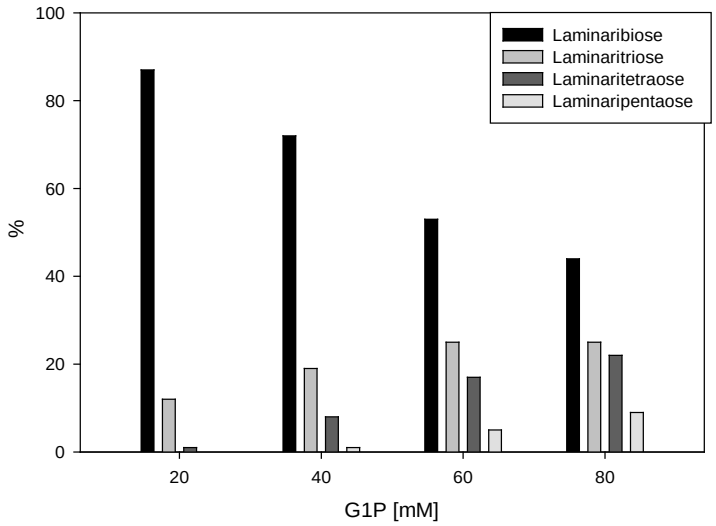


Fig.5.18 Percentage values of products obtained after 6 hour reaction with varying G1P concentrations. Initial reaction mixture with 20 mM Glc in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

The results reveal that increase in glucose-1-phosphate concentrations favor the formation of higher oligomers. This can be explained by the fact that when glucose is nearly consumed by LP, the enzyme will use glucose-1-phosphate and laminaribiose, instead of glucose, to form higher oligomers. Also the formation of higher oligomers is dependent on the glucose, laminaribiose, and laminaritriose concentrations. With regards to a trienzymatic system, this means that glucose-1-phosphate concentrations should either be kept at a minimum or laminaribiose needs to be removed from the reaction system. The latter is more favorable, as low glucose-1-phosphate concentrations will also result in lower laminaribiose formation rates (figure 5.17).

5.2.3.3 Modeling of LP

The kinetics was initially described as an ordered bi-bi mechanism (Kitaoka et al. 1993). In the work of Goldemberg (1966) and Kitaoka (1993), the reactions involved were viewed separately from each other and not regarded as an entire combined reaction. As seen above, LP catalyzes not only the production of laminaribiose, but also its higher oligomers. To fully describe the kinetic model, the two substrate kinetics (eq. 3.6) must be extended by the side reaction to the higher oligomers. The higher oligomers are of theoretical interest only, hence the modeling was restricted to formation of laminaribiose and consumption to laminaritriose (figure 5.19). The terms describing LP kinetics are stated in eq. 5.2-5.5.

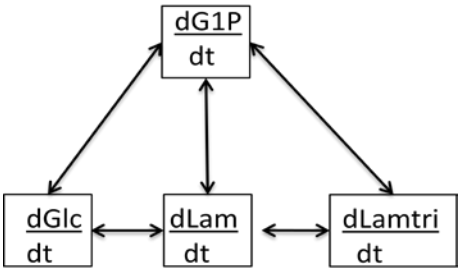


Fig.5.19 Kinetic reaction model to describe reaction mechanism of LP

$$\mathbf{v_{Lam}} = -\frac{\mathbf{dG1P}}{\mathbf{dt}} = -\frac{\mathbf{dGlc}}{\mathbf{dt}} = \mathbf{v_{maxLam}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} \cdot \frac{[\mathbf{Glc}]}{\mathbf{K_{MGlc}}+[\mathbf{Glc}]} - \mathbf{v_{maxLamtri}} \cdot \frac{[\mathbf{Lam}]}{\mathbf{K_{MLam}}+[\mathbf{Lam}]} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} = \frac{\mathbf{dLam}}{\mathbf{dt}} \quad \textbf{(5.2)}$$

$$\mathbf{v_{Lamtri}} = -\frac{\mathbf{dLam}}{\mathbf{dt}} = -\frac{\mathbf{dG1P}}{\mathbf{dt}} = \mathbf{v_{maxLamtri}} \cdot \frac{[\mathbf{Lam}]}{\mathbf{K_{MLam}}+[\mathbf{Lam}]} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} = \frac{\mathbf{dLamtri}}{\mathbf{dt}} \quad \textbf{(5.3)}$$

$$\frac{\mathbf{dG1P}}{\mathbf{dt}} = -\mathbf{v_{maxLam}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} \cdot \frac{[\mathbf{Glc}]}{\mathbf{K_{MGlc}}+[\mathbf{Glc}]} - \mathbf{v_{maxLamtri}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} \cdot \frac{[\mathbf{Lam}]}{\mathbf{K_{MLam}}+[\mathbf{Lam}]} \quad \textbf{(5.4)}$$

$$\frac{\mathbf{dGlc}}{\mathbf{dt}} = -\mathbf{v_{maxLam}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}}+[\mathbf{G1P}]} \cdot \frac{[\mathbf{Glc}]}{\mathbf{K_{MGlc}}+[\mathbf{Glc}]} \quad \textbf{(5.5)}$$

The model was used on a set of reactions with 5 different G1P concentrations and 5 Glc concentrations (table 5.6). Figure 5.20 shows an exemplary graph of experimental together with modeling results for one experiment.

Tab.5.6 Modeling of kinetic parameters for LP

K_{MG1P} [mM]	K_{MGlc} [mM]	K_{MLam} [mM]	v_{maxLam} [mM·min^{-1}]	$v_{maxLamtri}$ [mM·min^{-1}]	R^2 -
363	3.2	0.007	0.05	0.003	0.99

The model with a regression coefficient of 0.99 describes the reaction occurring very well. As can be seen from the results, the enzyme has a much higher affinity towards laminaritriose than the laminaribiose, as a much lower concentration is required to reach the laminaritriose half maximum reaction rate (0.007 mM) than for laminaribiose (3.2 mM). As G1P being the substrate for the formation of laminaribiose and higher oligomers, it possesses a relatively high K_{MG1P} of 363 mM. V_{Max} of laminaritriose formation is at 0.003 mM·min^{-1} while the maximum reaction rate for the formation of laminaribiose has a value of 0.05 mM·min^{-1}.

Literature suggests K_M values which do not align with the modeled data. Goldemberg (1966) calculated the kinetic parameters based on double substrate kinetics, for each reaction and subsequent reaction viewed independent from each other. The kinetics in this thesis is based on the (side) reactions having a direct influence on laminaribiose and its higher oligomers being formed. This makes it obvious, that a comparison between the modeled kinetic values with the data calculated in literature cannot be made.

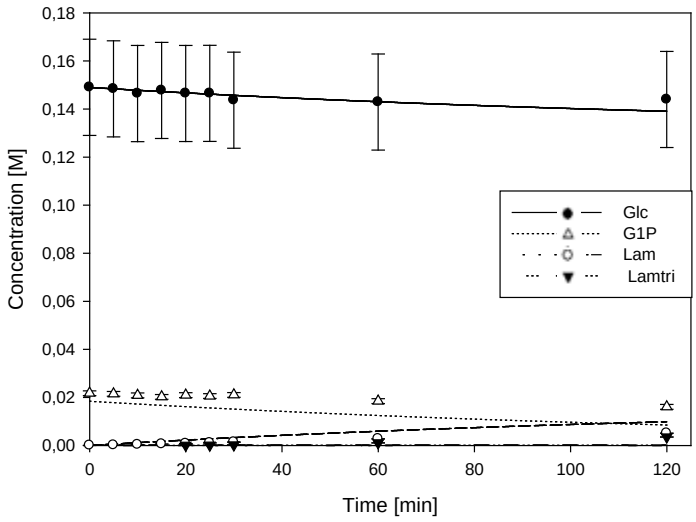


Fig.5.20 Exemplary Kinetics of laminaribiose and its subsequent reaction (lines show the modeling results).

Due to the results explaining the influence of glucose and glucose-1-phosphate on the formation of laminaribiose and it higher oligomers, it is interesting to determine what ratio of the two substrates ought to be used for high laminaribiose yields and while avoiding the subsequent reactions. Based on the modeling of the experiments, it can be seen that the higher the Glc/G1P ratio the more laminaribiose yields are obtained. Figure 5.22 shows exemplary models using 20 mM, 40 mM, and 60 mM glucose, while G1P kept at 20 mM.

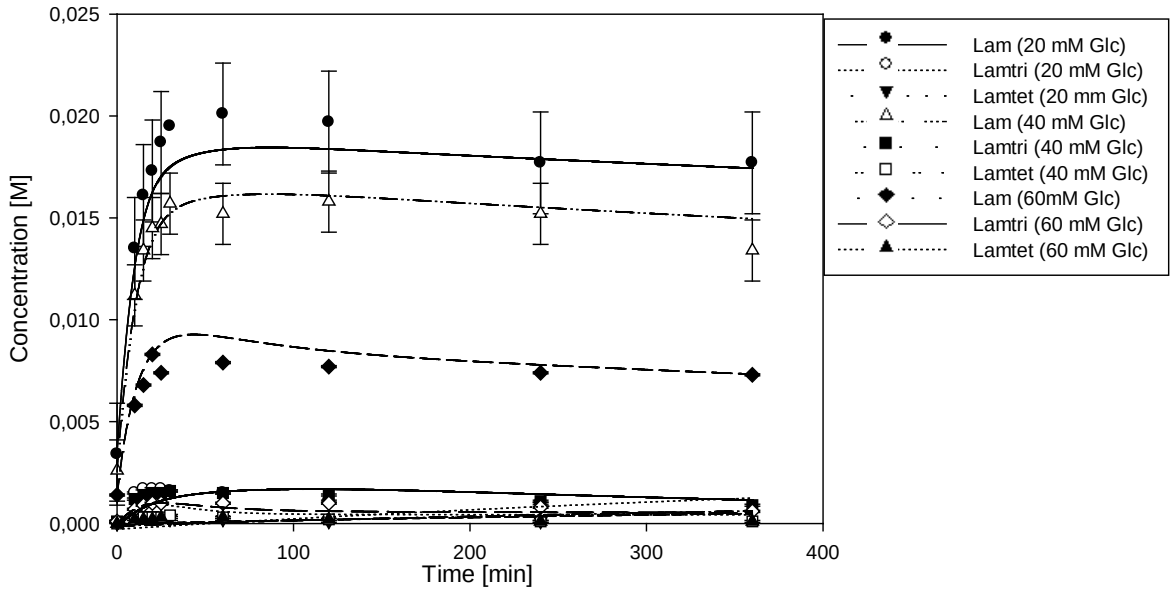


Fig.5.21 Exemplary Kinetics of laminaribiose and its subsequent reaction, using 20mM, 40 mM, and 60 mM Glc with 20 mM G1P. Lines show the modeling results

To get a better overview of how the ratio affects the products obtained, the modeled concentrations after 360 minutes are represented as percentages in relation to the total amount of products (figure 5.22).

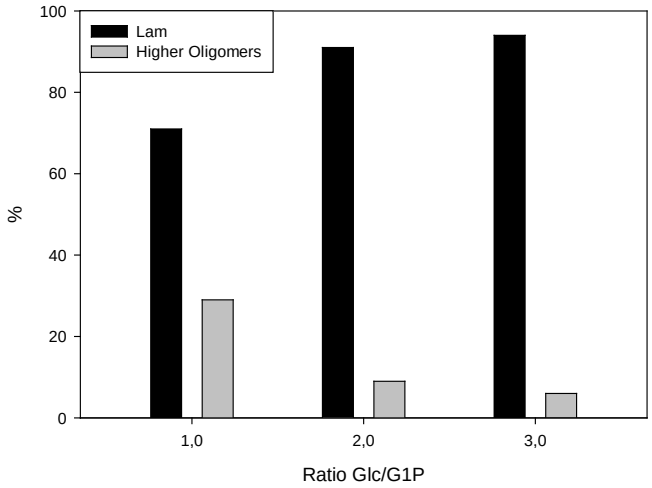


Fig.5.22 Percentage values of products obtained after 6 hour reaction with varying Glc/G1P ratios concentrations. Reactions were carried out in Sorensen buffer (pH 6.2, 500 mM) at 45°C.

When the ratio is at 1, a considerable increase of higher oligomers yield is observed with 72% laminaribiose and 28% higher oligomers. If Glc/G1P ratios increase, the laminaribiose yield continually increases. At a ratio of 3, the higher oligomers merely represent 6% of the products formed.

As the results indicate, a high Glc/G1P ratio will favor the laminaribiose production significantly, while a ratio below 2 will tend to shift the products obtained towards the higher oligomers.

In general it must be taken into consideration that LP possesses an overall low enzymatic activity, in addition with low cultivation yields. This may be problematic when trying to immobilize LP, which may result in even greater reduction in activity.

5.2.4 Summary of LP characterization

Analysis of the LP solution revealed that the solution co-exists as a monomer and dimer with molecular masses of approximately 120 kDa and 250 kDa respectively. Activity tests confirmed the production of laminaribiose and the side reactions to higher oligomers in form of laminaritriose and –tetraose (Kitaoka et al. 1991). Optimal working conditions were found between 42°C-45°C at pH 6.2. The pH determined is in accordance with the optimum found in literature (Goldemberg et al 1966).

For the kinetic investigation of the reaction, a reaction model was proposed, which enables the determination of the kinetic parameters for the enzyme. As a basis for the modeling of LP kinetics, a double substrate mechanism was used (Goldemberg et al. 1966), which was extended to include the

side reactions to higher oligomers. The obtained parameters showed that the subsequent reactions occur at a slower rate than laminaribiose production. The formation of laminaribiose and the subsequent reactions are highly dependent on the Glc/G1P ratios. Using Glc/G1P ratios smaller than 2, the higher oligomer production is favored. Ratios greater than 2 tend to shift the products towards laminaribiose, while the subsequent reactions are greatly reduced.

5.3 Characterization of sucrose phosphorylase

5.3.1 Analysis of SP solution

SP catalyzes the phosphorolysis of sucrose with phosphate to fructose and glucose-1-phosphate. The SP solution was kindly provided by Bitop AG. The enzyme activity of the enzyme is 14.7 U/ml with a specific activity of 3.5 U/mg protein and is comparable to the results found in literature (Abada et al. 2008). The protein concentration had a value of 13.5 g/l. The molecular weight of SP is between 54-60 kDa, determined by SDS-PAGE, and optimum enzymatic activity at a pH of 7.0, again matching very well with data determined in literature (Koga et al. 1991; Goedl et al. 2009).

5.3.2 Effect of temperature on the activity of SP

As already discussed (Chapter 5.2), the optimal temperature range for LP is at 42°C-45°C and significant activity loss at 50°C is observed. Hence, the activity of SP at temperatures beyond 50°C was not investigated, as all enzymes are to be combined in a trienzymatic system.

The optimal temperature within the range investigated was found between 45°C-50°C (figure 5.23). Kitao determined an optimal temperature of 42°C with a decrease in activity at 48 °C (Kitao et al. 1993), corresponding to slightly lower values than determined in this thesis. With the Arrhenius function, an activation energy with a value of 45 kJ/mol was calculated.

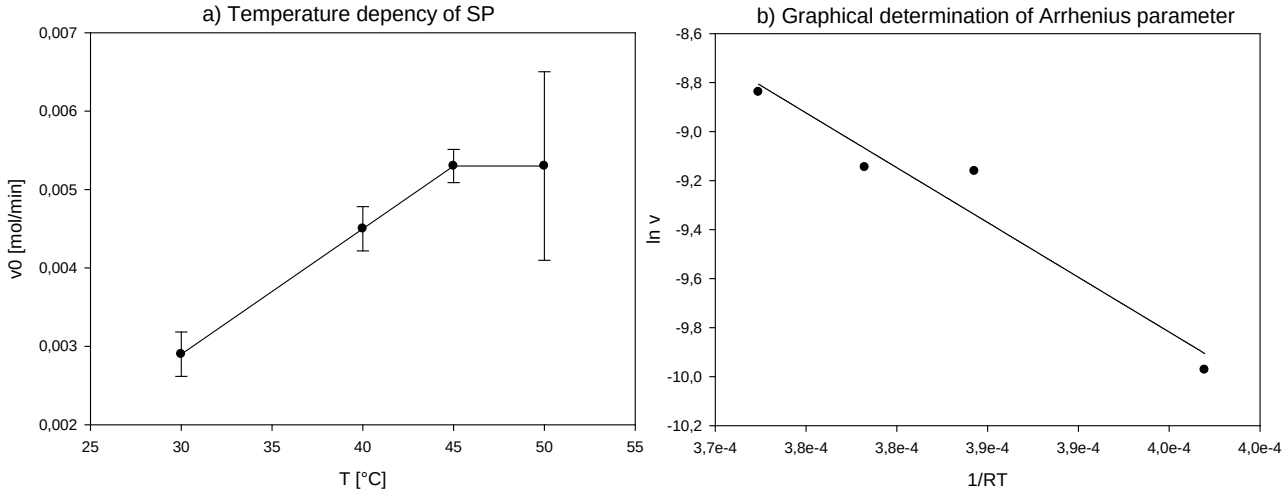


Fig.5.23 a) Temperature dependence of the initial reaction rates of sucrose phosphorylation by SP and b) Determination of Arrhenius parameter. Initial reaction mixture with 300 mM Suc in Sorensen buffer (pH 7)

5.3.3 Kinetic investigations and modeling of SP

For the synthesis of glucose-1-phosphate, sucrose (donor) and phosphate (acceptor) are needed. While carrying out phosphorolysis, it was observed that glucose-1-phosphate was not only produced, but decreased over time (figure 5.24). At the same time glucose concentrations increased.

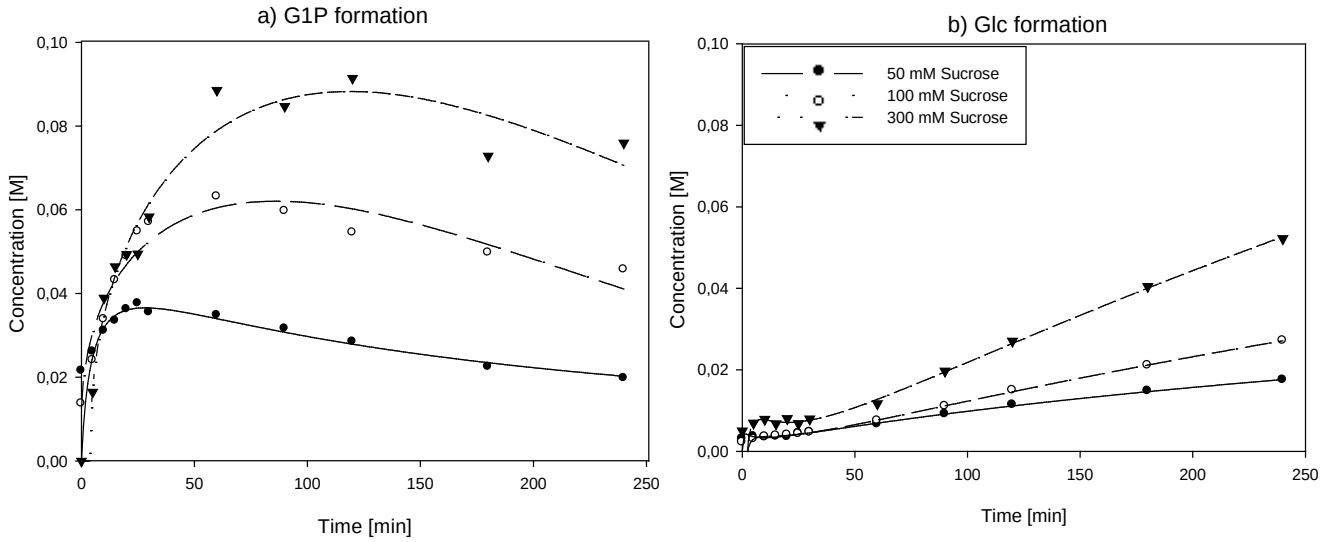


Fig.5.24 a) formation of G1P b) formation of Glc. Initial reaction mixture with in Sorensen buffer (pH 7.0) at 45°C

Therefore it becomes evident that SP is also able to hydrolyze G1P into glucose and phosphate. It has been shown that in the absence of an acceptor, SP catalyzes an exchange of phosphate between G1P and inorganic phosphate (Silverstein et al. 1966). The first reaction step of SP is the reversible cleavage of the phosphate bond. The assumption of a hydrolysis of G1P was tested by carrying out a reaction with SP, containing only G1P and inorganic phosphate. The experiments revealed a hydrolysis of G1P over time and comply with the data found in literature (figure 5.25) (Silverstein et al. 1966, Cohn 1949). Compared with the initial reaction rate of the phosphorolysis, hydrolysis occurs approximately at a 50% lesser rate. Due to the hydroylsis, a competitive inhibition behavior between sucrose and G1P will occur.

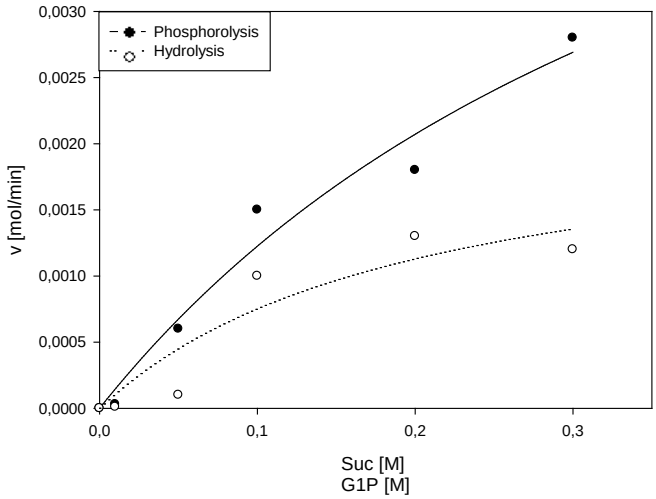


Fig.5.25 Comparison of phosphorolysis of sucrose and hydrolysis of G1P with SP. Phosphorolic rate of SP in Sorensen buffer (pH 7) and Suc, Hydrolytic rate of SP in Sorensen buffer (pH 7) and G1P at 45°C

It is known for SP to react under a ping-pong mechanism (Silverstein et al. 1966, Taylor et al. 1982, Goedl et al. 2008). The double substrate kinetics was used as a starting point for modeling the reaction (eq. 5.6).

$$\mathbf{v} = \mathbf{v_{max}} \cdot \frac{[\mathbf{Suc}][\mathbf{P}]}{\mathbf{K_{MSuc}} \cdot [\mathbf{P}] + \mathbf{K_{MP}} \cdot [\mathbf{Suc}] + [\mathbf{Suc}][\mathbf{P}]} \qquad \textbf{(5.6)}$$

However based on the fact that phosphorolysis and hydrolysis occur alongside, the double substrate kinetics does not fully apply. The terms used to describe SP kinetics and its connections is shown in figure 5.26. The formation of G1P and Fru are described by the green lines. As experiments revealed G1P is hydrolyzed by SP alongside phosphorolysis and is represented in red.

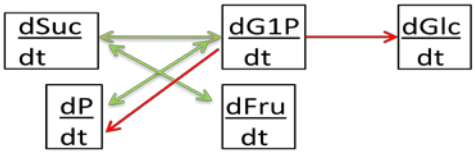


Fig.5.26 Kinetic reaction model to describe reaction mechanism of SP

The kinetic reaction model for formation of glucose-1-phosphate can be described by two terms (eq. 5.7).

$$\frac{\mathbf{dG1P}}{\mathbf{dt}} = \text{"}\mathbf{Acceptor}\text{"} - \text{"}\mathbf{Hydrolysis}\text{"} \qquad \textbf{(5.7)}$$

1) Acceptor reaction

Basically the phosphorolysis conforms to a double-substrate-kinetics (eq. 5.6) (Silverstein et al. 1966). As seen the reaction consists of a competitive inhibition behavior due to G1P hydrolysis, which must be considered in the model (eq. 5.8, K_{InhG1P}). By adding phosphate in excess it could be reduced to monosubstrate type kinetics (eq. 5.9).

$$\mathbf{v_{Acc}} = -\frac{\mathbf{dSuc}}{\mathbf{dt}} = -\frac{\mathbf{dP}}{\mathbf{dt}} = \mathbf{v_{maxSuc}} \cdot \frac{[\mathbf{Suc}]}{\mathbf{K_{MSuc}} + [\mathbf{Suc}] + \frac{\mathbf{K_{MSuc}}}{\mathbf{K_{InhG1P}}} \cdot [\mathbf{G1P}]} \cdot \frac{[\mathbf{P}]}{\mathbf{K_{MP}} + [\mathbf{P}]} = \frac{\mathbf{dG1P}}{\mathbf{dt}} = \frac{\mathbf{dFru}}{\mathbf{dt}} \qquad \textbf{(5.8)}$$

$$\mathbf{v_{Acc}} = -\frac{\mathbf{dSuc}}{\mathbf{dt}} = \mathbf{v_{maxSuc}} \cdot \frac{[\mathbf{Suc}]}{\mathbf{K_{MSuc}} + [\mathbf{Suc}] + \frac{\mathbf{K_{MSuc}}}{\mathbf{K_{InhG1P}}} \cdot [\mathbf{G1P}]} = \frac{\mathbf{dG1P}}{\mathbf{dt}} = \frac{\mathbf{dFru}}{\mathbf{dt}} \qquad \textbf{(5.9)}$$

2) Hydrolysis

Hydrolysis is represented by the following equation (eq. 5.10), again extended with a competitive inhibition caused by sucrose (K_{InhSuc}).

$$\mathbf{v_{Hyd}} = -\frac{\mathbf{dG1P}}{\mathbf{dt}} = \mathbf{v_{maxG1P}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}} + [\mathbf{G1P}] + \frac{\mathbf{K_{MG1P}}}{\mathbf{K_{InhSuc}}} \cdot [\mathbf{Suc}]} = \frac{\mathbf{dP}}{\mathbf{dt}} = \frac{\mathbf{dGlc}}{\mathbf{dt}} \qquad \textbf{(5.10)}$$

Therefore the hydrolysis reaction must be added to the acceptor reaction (eq. 5.8) to fully describe the model for SP (eq. 5.11).

$$\frac{\mathbf{dG1P}}{\mathbf{dt}} = \mathbf{v_{Acc}} - \mathbf{v_{maxG1P}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}} + [\mathbf{G1P}] + \frac{\mathbf{K_{MG1P}}}{\mathbf{K_{InhSuc}}} \cdot [\mathbf{Suc}]} = -\frac{\mathbf{dP}}{\mathbf{dt}} \qquad \textbf{(5.11)}$$

The model was used on a set of reactions with 6 different sucrose concentrations. Figure 5.27 shows an exemplary graph of experimental together with modeling results for one experiment. It can be seen that the reaction model fits very well to the experimental data.(table 5.7).

Tab.5.7 Modeling of kinetic parameters for SP

K_{MSuc} [mM]	K_{MG1P} [mM]	K_{InhG1P} [mM]	K_{InhSuc} [M]	v_{maxSuc} [mM·min^{-1}]	v_{maxG1P} [mM·min^{-1}]	R^2 -
0.7	$5 \cdot 10^{-5}$	2.8	5	30	0.1	0.95

The model calculated for v_{maxSuc} in terms of phosphorolysis 30 mM/min and for hydrolysis 0.1 mM/min (v_{maxG1P}). Therefore, hydroylsis can be neglected, as the phosphorolysis takes place at a 300x faster rate. The extremely small K_{MG1P} may indicate an inaccuracy in the modeling results and can be regarded as an reaction of zero order. It can be deduced that hydrolysis is favored at low sucrose concentrations. G1P will eventually reach concentrations surpassing the original substrate and therefore promoting the hydrolysis and glucose production.

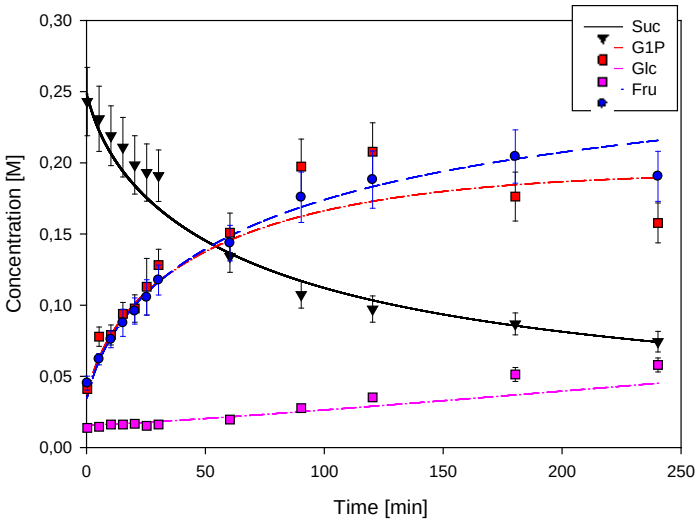


Fig.5.27 Kinetics of G1P produced and hydrolyzed (lines show the modeling results). Final reaction volume: 3 ml: 250 mM Suc in Sorensen buffer (pH 7), temperature 45°C, and reaction time 4 h

5.3.4 Summary of SP characterization

Activity tests confirmed the reaction of sucrose and inorganic phosphate to fructose and glucose-1-phosphate. Optimal reaction conditions for SP were found at a temperature of 45°C-50°C at pH 7.0. For modeling the reaction, a ping-pong mechanism was used as a basis (Goedl et al. 2008) and extended with a hydrolysis term, as glucose-1-phosphate is also a substrate for SP, and a competitive inhibition term for both sucrose and G1P. Reaction model matches very well with the concentration gradients of acceptor and hydrolysis reaction. Modeling results indicate that phosphorolysis occurs at such high rates, that the hydrolysis rate can be neglected. Furthermore, the model suggests that low substrate concentrations cause a shift from product formation to hydrolysis.

5.4 Characterization of glucose isomerase

GI catalyzes the isomerization between fructose and glucose. Since this enzyme is already well studied only few confirming experiments were done (Bhosale et al. 1996, Straatsma et al. 1983). Temperature optimum is found between 60°C-80°C with a pH optimum of 8.5 (Bhosale et al. 1996). The provided GI has an activity 150 U/mg. With regards to the optimal temperature for LP (42°C-45°C) and the establishment of a trienzymatic system, the reaction temperature for GI experiments was set to 45°C. GI can be described by the following mechanism (Converti and Del Borghi 1998) (eq. 5.12).

$$G + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} F + E \qquad \textbf{(5.12)}$$

G: Glc
F: Fru
E: free enzyme
X: Intermediate complex between enzyme and glucose (EG) or fructose (EF) respectively
$k_{\pm x}$: rate constants of elementary reactions

By applying the pseudo-steady-state hypothesis for X, this approach leads to the Michaelis-Menten-type equation (eq. 3.5). The reaction can be modeled as an equilibrium reaction between glucose and fructose using two Michaelis-Menten-kinetics (eq. 5.13).

$$-\frac{\mathbf{d_{Fru}}}{\mathbf{dt}} = \left(\frac{\mathbf{v_{maxFru}} \cdot \mathbf{Fru}}{\mathbf{K_{Fru}} + \mathbf{Fru}}\right) - \left(\frac{\mathbf{v_{maxGlc}} \cdot \mathbf{Glc}}{\mathbf{K_{Glu}} + \mathbf{Glc}}\right) = \frac{\mathbf{d_{Glc}}}{\mathbf{dt}} \qquad \textbf{(5.13)}$$

The model was used on a set of reactions with 6 different fructose concentrations. Figure 5.28 shows an exemplary graph of the experimental and modeling results for isomerization of fructose.

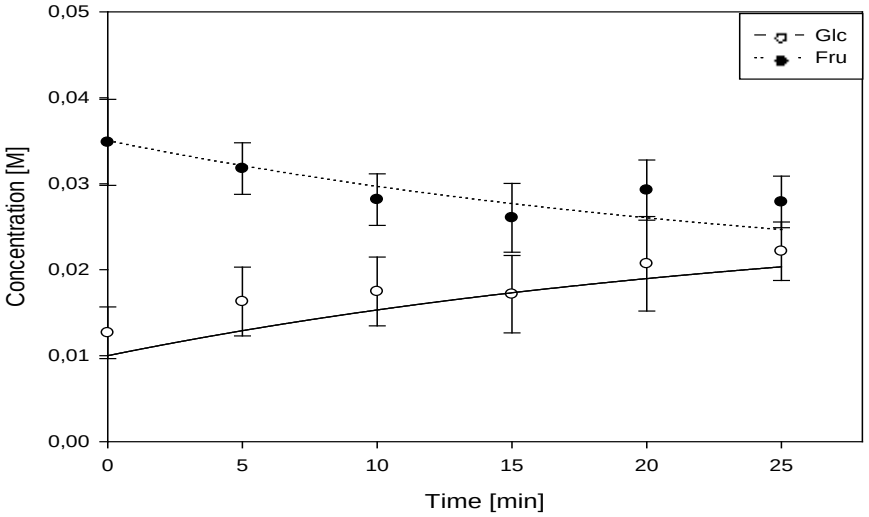


Fig.5.28 Kinetics of Glc produced through isomerization (lines show the modeling results). Reaction mixture contained, in a final volume of 3 ml, 35 mM fructose, temperature 45°C, and reaction time 25 min

By modeling the affinity constants were calculated as K_{Fru} = 300 mM and K_{Glu} = 360 mM, obtaining affinity constants with similar values as found in literature (Suekane et al. 1978, Jenkins et al. 1992, Inyang et al. 1995). v_{maxFru} is 6.3 mM·min^{-1} and v_{maxGlc} 2.9 mM·min^{-1} with a regression coefficient of 0.98.

Alternatively, a native, enzymatic solution of GI was used which was kindly provided by Novozymes. The kinetic parameters were modeled to have values of K_{Fru} = 457 mM and K_{Glu} = 400 mM. v_{maxFru} is 1.5 mM·min^{-1} and v_{maxGlc} 2 mM·min^{-1} with an R^2 value of 0.95. The enzymatic solution has a decreased activity in comparison with the GI granules. This is due to the stabilizing effect of the granules and the addition of Mg^{2+} to the granules as an activator for GI (Jorgensen et al. 1988).

5.4.1 Summary of GI characterization

Even though the temperature optima for GI as stated in literature (Jorgenson et al. 1988) were not used in this work, an appealing activity of the enzymes at 45°C was still observed, with regards to the temperature optima for the other enzymes used in this thesis.

The reaction was modeled as a reversible mechanism, and by applying a pseudo-steady-state hypothesis for the intermediate complex between GI and glucose/fructose (Converti and Del Borghi 1998).

5.5 Establishment of a native bienzymatic reaction system

After separate characterization of all three enzymes, SP and GI were first combined to establish a bienzymatic reaction system, for the production of the intermediates needed for LP to eventually produce laminaribiose.

5.5.1 Effect of temperature on the native bienzymatic system

A temperature must be found which is suitable for a combined use of both SP and GI. The maximum yields of G1P in the bienzymatic system were compared to the monoenzymatic reaction (figure 5.29). G1P rises with increasing temperature, with an optimum between 42°C-45°C and corresponds to the observations made for the monoenzymatic reaction.

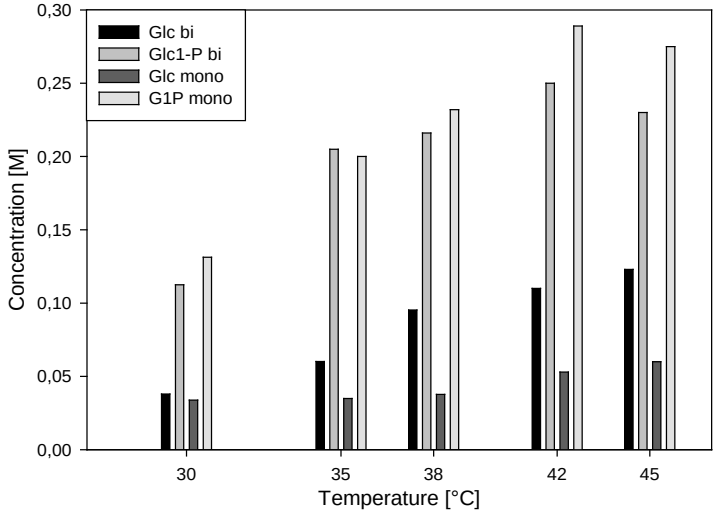


Fig.5.29 Comparison of G1P in mono-/bienzymatic systems from 30°C-45°C after 2 hours reaction time. Initial reaction mixture with 300 mM Suc in Sorensen buffer (pH 7.0)

Interestingly, G1P yields for the monoenzymatic system are slightly higher than in the bienzymatic reaction. This is due to an inhibition caused by glucose (Doudoroff 1943), which is produced by the isomerization of fructose with GI. The higher the glucose concentrations, the stronger the inhibiting effect. This effect must be considered for modeling the reaction in a bi- and later trienzymatic system, by extending equation 5.9 with the following term highlighted in grey.

$$-\frac{\mathbf{dSuc}}{\mathbf{dt}} = \mathbf{V_{maxSuc}} \cdot \frac{[\mathbf{Suc}]}{\mathbf{K_{MSuc}} \cdot \left(1+\frac{[\mathbf{Glc}]^2}{\mathbf{K_{InhGlc}}}\right)+[\mathbf{Suc}]+\frac{\mathbf{K_{MSuc}}}{\mathbf{K_{InhG1P}}} \cdot [\mathbf{G1P}]} \qquad \textbf{(5.14)}$$

With rising temperature, the initial reaction rates for fructose conversion will increase, producing more glucose in a shorter amount of time (figure 5.30).

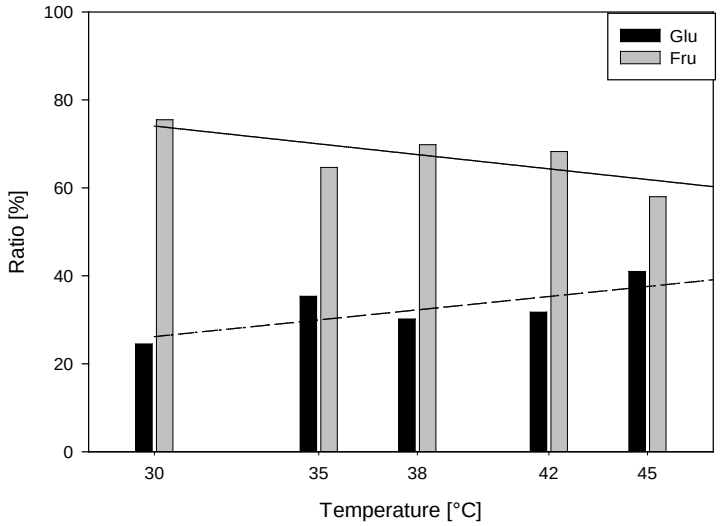


Fig.5.30 Comparison of Glc/Fru ratio in bienzymatic systems from 30°C-45°C after 2 hours reaction time. Initial reaction mixture with 300 mM Suc in Sorensen buffer (pH 7.0)

As can be seen in the graph, the Glc/Fru ratio at 30°C has a value of 22:78. With increasing temperature the ratio becomes smaller reaching 41:58 at a temperature of 45°C after 2 hours reaction time. The results indicate an operating temperature for a bienzymatic system is found at 45°C. Both enzymes are active, with SP being at its optimum and GI producing satisfying glucose concentrations.

5.5.2 Kinetics of native bienzymatic reaction

The terms used to describe SP and GI kinetics in the bienzymatic reaction and their connections are shown in figure 5.31.

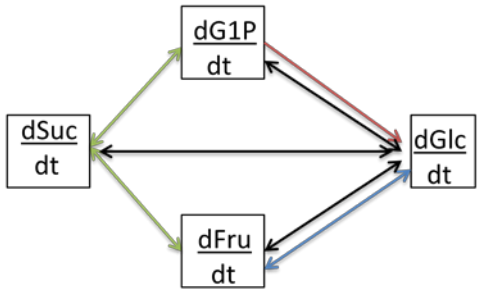


Fig.5.31 Kinetic model for bienzymatic reaction with SP/GI

The reaction is described by the combination of acceptor reaction, hydrolysis, competitive inhibition caused by glucose and isomerization of fructose (eq. 5.15-5.17). The influences between the differential equations are indicated by: green arrows describing the formation of G1P and Fru; blue arrows representing the isomerization; black arrows showing competitive inhibition of glucose. Hydrolysis is visualized with red arrows. The decline of phosphate was again neglected because of adding it in excess.

$$\frac{\mathbf{dG1P}}{\mathbf{dt}} = \mathbf{V_{Acc}} - \mathbf{V_{maxG1P}} \cdot \frac{[\mathbf{G1P}]}{\mathbf{K_{MG1P}} + [\mathbf{G1P}] + \frac{\mathbf{K_{MG1P}}}{\mathbf{K_{InhSuc}}} \cdot [\mathbf{Suc}]} \quad \textbf{(5.15)}$$

$$\frac{dFru}{dt} = V_{maxSuc} \cdot \left(\frac{[Suc]}{K_{MSuc} \cdot \left(1 + \frac{[Glc]^2}{K_{InhGlc}}\right) + [Suc] + \frac{K_{MSuc}}{K_{InhG1P}} \cdot [G1P]} \right) - V_{maxFru} \cdot \left(\frac{[Fru]}{K_{MFru} + [Fru]} \right) + V_{maxGlc} \cdot \left(\frac{[Glc]}{K_{MGlc} + [Glc]} \right) \quad (5.16)$$

$$\frac{dGlc}{dt} = V_{maxG1P} \cdot \left(\frac{[G1P]}{K_{MG1P} + [G1P] + \frac{K_{MG1P}}{K_{InhSuc}} \cdot [Suc]} \right) + V_{maxFru} \cdot \left(\frac{[Fru]}{K_{MFru} + [Fru]} \right) - V_{maxGlc} \cdot \left(\frac{[Glc]}{K_{MGlc} + [Glc]} \right) \quad (5.17)$$

The model was used on a set of reactions with 3 different sucrose concentrations. Figure 5.32 shows an exemplary graph of the experimental and modeling results of one of the kinetic reactions. The developed reaction model fits the experimental data very well. The overall regression coefficient of all the kinetic reactions of bienzymatic reactions is 0.97 (table 5.8). For a better comparison the second row in table 5.8 represents the parameters determined for the monoenzymatic systems.

Tab.5.8 Modeling of kinetic parameters for bienzymatic system using SP/GI with sucrose as substrate

	K_{MSuc}	K_{MG1P}	K_{MFru}	K_{MGlc}	K_{InhG1P}	K_{InhSuc}	K_{InhGlc}	v_{maxSuc}	v_{maxG1P}	v_{maxFru}	v_{maxGlc}	R^2
			[mM]			[mM]			[mM·min^{-1}]			-
Bi	1.1	$1.2 \cdot 10^{-5}$	180	224	0.5	4000	273	11	3	2.1	4	0.97
Mono	0.7	$5 \cdot 10^{-5}$	300	360	2.8	5000		30	0.1	6.3	2.9	0.98

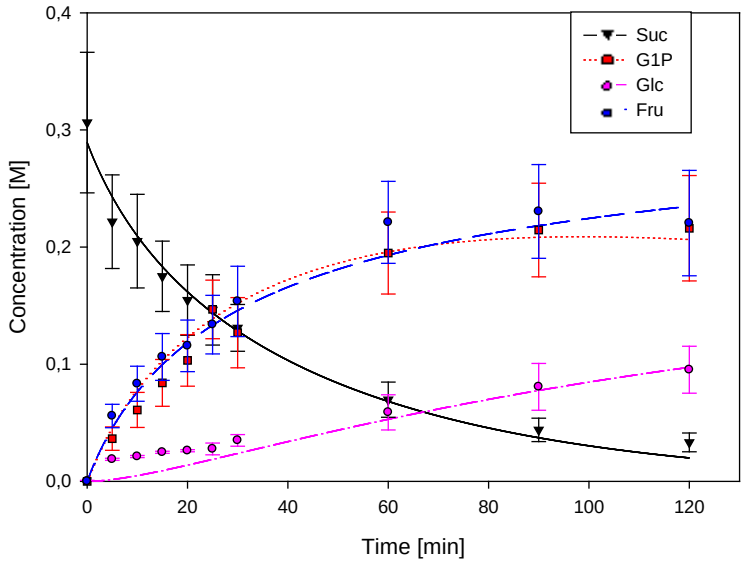


Fig.5.32 Kinetics of bienzymatic reaction system (lines show the modeling results). Final reaction volume: 3 ml, 300 mM Suc in Sorensen buffer (pH 7), temperature 38°C, and reaction time 2 h

Values of K_{MSuc} and K_{MG1P} are very similar to the monoenzymatic values, with K_{MSuc} slighty increased. This was to be expected due to the competitive inhibition caused by glucose (Doudoroff 1943). V_{maxSuc} was reduced to approximately half its value in the monoenzymatic system (30 mM/min) and cannot be explained by inhibition. V_{maxG1P} is increased from 0.1 mM/min to 3 mM/min, which cannot yet be explained.

As can be seen from the modeling results, the inhibition of phosphorolysis will be caused by glucose concentrations with a $K_{MInhGlc}$ of 270 mM. This inhibition, however, should be easily circumvented when implementing laminaribiose phosphorylase, as it uses glucose-1-phosphate and glucose to

produce laminaribiose. Therefore glucose would be kept below 270 mM, which then does not have an inhibitory effect on SP anymore. Nearly no inhibitory effect caused by sucrose will take place.

As with the bienzymatic system K_{InhG1P} has an extremely small value (0.5 mM). This would imply that only concentrations below 0.5 mM can be used for using LP for the production of laminaribiose. This would result in an extremely small reaction rate, as the K_M value for LP is 363 mM. Whether or not this applies on an actual reaction, will be clarified when a trienzymatic system has been established.

5.5.3 Summary of bienzymatic system establishment

For getting more information about consequences of combined use of the enzymes, firstly the combination of SP and GI were studied.

As seen in literature (Jorgensen et al. 1988), the optimum for GI is at temperatures beyond 60°C, which however denaturizes SP (Kitao et al. 1993). Therefore, a temperature must be found which is suitable for a combined use of both SP and GI. Results indicate an ´optimum temperature´ for operating an bienzymatic system is found at 45°C. Both enzymes are active, with SP being at its optimum and GI producing satisfying glucose isomerizing reaction rates.

However, slightly decreased G1P amounts are obtained due to an inhibition of SP. The inhibition is caused by glucose (Doudoroff 1943). This has to be taken into consideration for modeling the bienzymatic reaction. The concentration gradients of substrates and intermediate products for LP fit very well to the model with a regression coefficient of 0.97. Glucose greatly affects the inhibition of SP at high concentrations. Hence, it is important to keep glucose concentrations low, which should be realized when LP is added to the reaction system.

5.6 Establishment of a native trienzymatic reaction system

After establishment of a bienzymatic system using GI and SP to produce the intermediates needed for production of laminaribiose, the trienzymatic system was studied.

An activity test was carried out to obtain basic information about the entire system (figure 5.33).

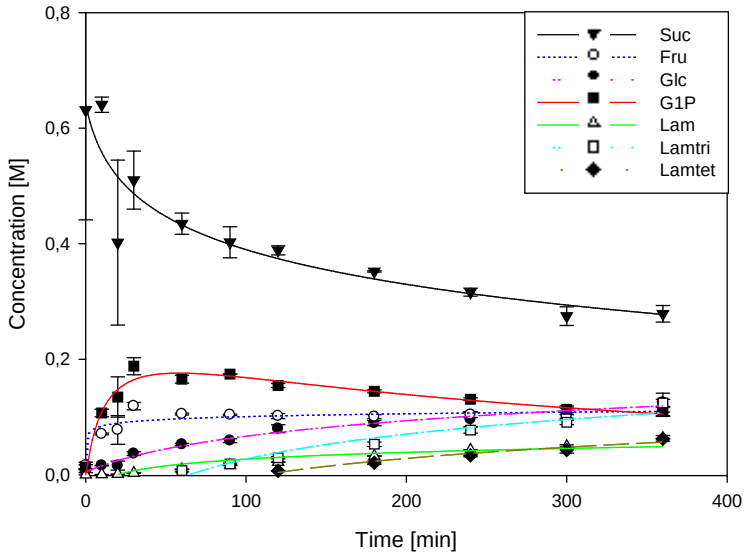


Fig.5.33 Trienzymatic reaction using native SP/GI/LP. Final reaction volume: 3 ml, 600 mM Suc in Sorensen buffer (pH 6.2), temperature 38°C, and 6 hours reaction time.

All the three enzymes obviously retained their activity when combined into a reaction mixture of 600 mM sucrose. G1P consumption into laminaribiose is dominant compared to G1P formation, leading to a decrease of G1P concentration after 60 minutes. Fructose as a byproduct of G1P formation, is isomerized into glucose. Throughout the reaction the glucose concentrations are at a low level, due to the direct consumption to laminaribiose formation. G1P and glucose are converted into laminaribiose, which continuously rises in concentration. After 360 minutes the concentration of laminaribiose reached 100 mM. Comparable attempts in literature achieved a concentration of 110 mM after 48 hours incubation (Kitaoka et al. 1993b). The production of laminaritriose and laminaritetraose surpasses the formation rate of laminaribiose. This is not favorable, as the main goal is the yield and isolation of laminaribiose. In the trienzymatic system used by Kitaoka, laminaritriose was produced with a yield of 22 mM after 15 hours. The oligomers yields were also significantly higher than in Kitaoka´s system, where only trace amounts were detected. This implies that the preliminary system proposed in this thesis already synthesizes higher product yields than in Kitaoka´s work, which used an imidazole-HCl buffer at pH 7.0 at 37°C and 48 hours reaction time. Furthermore the cultivation used by Kitaoka yielded a lower activity of LP than obtained in this thesis (see also chapter 5.1), causing a lower laminaribiose formation rate.

As Sorensen buffer was used in the trienzymatic system, the kinetic parameters of GI and LP were determined and compared to the unbuffered media. The model for GI was used on a set of reactions with 6 different fructose concentrations. The kinetic parameters for GI overall showed an increase in affinity, while maximum reaction rates stayed relatively constant when using a buffered system (table 5.9). This is most likely caused by a stabilizing effect the buffer applies onto the enzyme.

Tab.5.9 Comparison of K_M und v_{max} for GI in unbuffered and buffered system

	K_{Fru} mM	K_{Glu} mM	v_{maxFru} mM·min^{-1}	v_{maxGlc} mM·min^{-1}	R^2
unbuffered	300	360	6.3	2.9	0.98
buffered	230	170	5	3	0.98

The model for LP was used on a set of reactions with 5 different G1P concentrations and 5 Glc concentrations. In terms of LP kinetics the enzymatic activity overall stayed relatively constant (table 5.10).

Tab.5.10 Comparison of kinetic parameters for LP in monoenzymatic and trienzymatic conditions.

	K_{MGlc} [mM]	K_{MG1P} [mM]	K_{Lam} [mM]	v_{maxLam} [mM·min^{-1}]	$v_{maxLamtri}$ [mM·min^{-1}]	R^2 -
unbuffered	363	3.2	0.007	0.05	0.003	0.99
buffered	400	4	0.005	0.03	0.005	0.93

5.6.1 Effect of temperature on the native trienzymatic system

In order to optimize the yield of laminaribiose, the temperature was varied from 35°C-60°C (figure 5.34). The highest formation rate of laminaribiose is achieved at 45°C with an initial Lam reaction formation rate of 0.3 mM/min. This corresponds to the monoenzymatic system of LP (figure 5.13) and hence no loss in activity takes place when LP is inserted into the trienzymatic reaction. A comparison with the trienzymatic systems of other works is not possible, due to the fact that this reaction process was only found to be carried out by Kitaoka who merely used a temperature of 37°C, to confirm the possibility of producing laminaribiose with all three enzymes (Kitaoka et al. 1993b). Due to different conditions used for determining the kinetic parameters (lower concentrations of enzymes used, lower temperature, enzymatic suspensions from different cultivations, resulting in different activities) the initial reaction rates for determining the effect of temperature should not be compared to the values determined in the kinetic modeling.

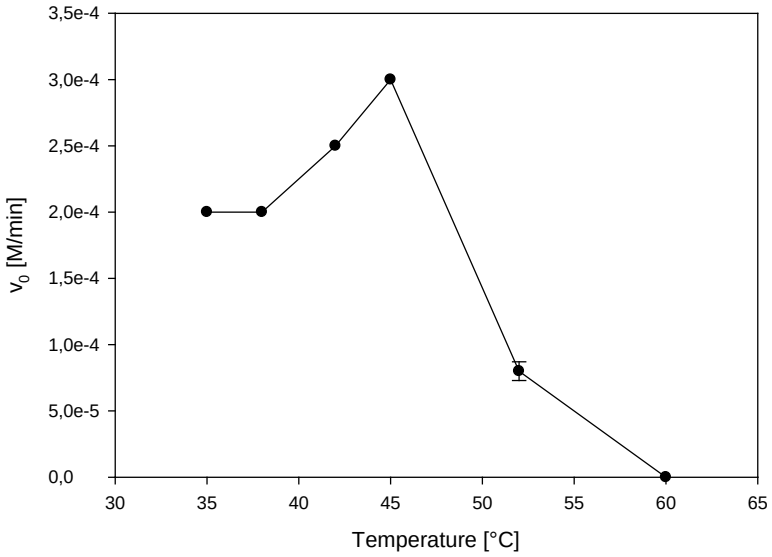


Fig.5.34 Influence of temperature on laminaribiose production in the trienzymatic system (600 mM Suc and 600 mM Sorensen buffer (pH 7))

At temperatures above 45°C, enzyme denaturation becomes a decisive factor and therefore a rapid decrease of the reaction rate of glucose-1-phosphate and laminaribiose (and its higher oligomers) formation is observed. Merely an increase of glucose formation will be observed, since GI approaches its optimum with rising temperature. For further investigations the system ought to be carried out at 45°C.

5.6.2 Effect of pH on the native trienzymatic system

The pH of the Sorensen buffer was varied and adjusted to the optimum of any of the three enzymes (figure 5.35) (pH 6.2 for LP (Goldemberg et al. 1966), 7.0 for SP (Silverstein et al. 1967), and 8.5 for GI (Bhosale et al. 1996).

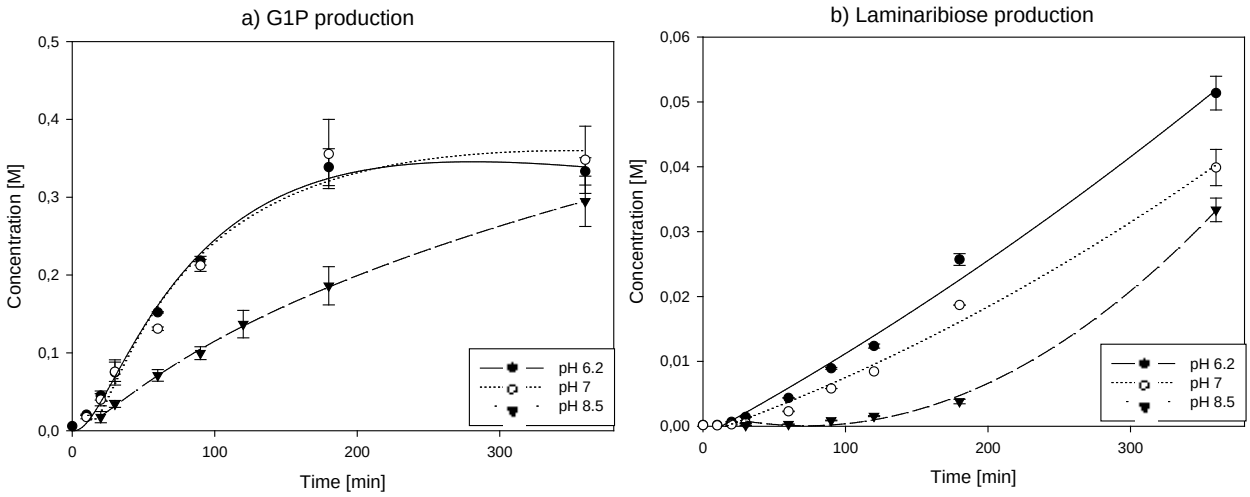


Fig.5.35a/b Effect of pH on the trienzymatic system with native enzymes a) Production of G1P and b) production of laminaribiose (500 mM Suc and 600 mM Sorensen buffer at 45°C)

Highest rates of laminaribiose production were achieved at pH 6.2 corresponding to the optimum for LP (Goldemberg et al. 1966). By increasing the pH, the formation rate of both G1P and laminaribiose

decreases. G1P production data show nearly identical rates at pH 6.2 and 7.0, but significantly reduced at pH 8.5. Hence it can be deduced that SP needs slightly acidic pH for optimal activity. After 200 minutes a decrease of G1P concentration is shown for pH 6.2. This may be caused by an increased activity of LP, causing a faster consumption of G1P at this pH than at pH 7.0.

The isomerization occurred in similar rates for all pH values, therefore the pH does not influence the enzymatic activity of GI.

In Kitaoka´s trienzymatic system, the reactions were carried out at a pH 7.0, in which all enzymes were still active (Kitaoka et al. 1993b). As results in this thesis revealed, by changing the pH to 6.2 the synthesis of laminaribiose using laminaribiose phosphorylase, glucose isomerase, and sucrose phosphorylase was greatly improved, yielding 25% more product than at pH 7.0 (figure 5.35b).

5.6.3 Kinetics of native trienzymatic reaction

The terms used to describe the trienzymatic reaction include, LP, SP, and GI kinetics. Their connections are shown in figure 5.36. The formation of G1P and Fru are described by the green lines. Hydrolysis of G1P is represented in red, blue arrows representing the isomerization; black arrows showing competitive inhibition of glucose. Orange lines represent the reaction of laminaribiose and purple lines symbolize the subsequent reactions to higher oligomers by LP.

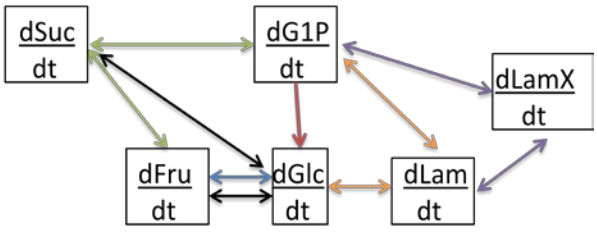


Fig.5.36 Kinetic reaction model to describe trienzymatic reaction system

Using the model, equations 5.15-5.17 and 5.2-5.5 have been combined. The model was used on a trienzymatic reaction lasting for 360 minutes (table 5.11).

Tab.5.11 Modeling of kinetic parameters for trienzymatic system

K_{MSuc}	K_{MG1P}	K_{MFru}	K_{MGlc}	K_{MLam}	K_{InhG1P}	K_{InhSuc}	K_{InhGlc}	v_{maxSuc}	v_{maxG1P}	v_{maxFru}	v_{maxGlc}	v_{maxLam}	$v_{maxLamtri}$	R^2
[mM]					[mM]			[mM·min^{-1}]						
1.6	$8 \cdot 10^{-9}$	156	540	$5 \cdot 10^{-9}$	0.4	3600	200	15	0.1	2.4	3.1	0.9	0.003	0.97

Based on the results, the models created for the single enzymes also apply for a combination in the trienzymatic system with a regression coefficient of 0.97. The modeling results fit the experimental data very well (figure 5.37). The kinetic parameters determined for the bienzymatic system are nearly the same as in the modeled trienzymatic reaction. However, it must be stated that K_M for G1P and Lam, cannot be regarded as accurate results, as they showed calculated value of 10^{-9} mM, which would

suggest an extremely high affinity towards the mentioned products, which is very unrealistic based on the modeling with the monoenzymatic reactions. It is likely that the set of substrate concentration was too little to give more accurate modeling results. Therefore more variations on substrate concentration should be carried out, to obtain more reasonable data.

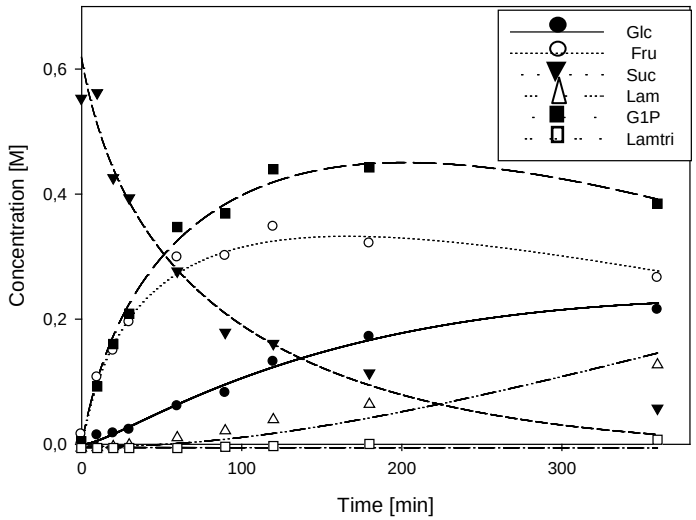


Fig.5.37 Kinetics of trienzymatic reaction system (lines show the modeling results). Final reaction volume: 3 ml, 600 mM Suc in Sorensen buffer (pH 6.2), temperature 42°C, and 360 minutes reaction time

The model suggests a reaction rate in which the consumption of Lam will eventually overcome its formation at later stages of the reaction.

5.6.4 Summary of trienzymatic establishment

In activity tests for the establishment of a trienzymatic system SP, GI, and LP retained their activity after being combined in a reaction mixture containing sucrose and Sorensen buffer. As a result, the produced intermediates G1P and Glc were consumed by LP to produce laminaribiose. The native trienzymatic system showed an improvement of laminaribiose yield by 25% when compared to the work of Kitaoka (Kitaoka et al. 1993b).

The subsequent reactions producing laminaritriose and laminaritetraose were also observed and surpassed laminaribiose formation rate. This makes it obvious, that an ISPR system needs to be eventually established, to remove laminaribiose from the reaction mixture.

For the trienzymatic system the optimal conditions for laminaribiose formation are at a temperature of 45°C and a pH of 6.2.

The combination of the mono-/bienzymatic kinetics led to an accurate description of the trienzymatic system, showing a similar trend as the experimental data.

5.7 ISPR for production and purification of laminaribiose

5.7.1 Preliminary investigations of ISPR with native trienzymatic reaction system

The trienzymatic system is successfully established and optimized, with good yields of laminaribiose. As seen previously, the side reactions also occurred with the production of laminaroligomers. In order to isolate the product and minimizing the subsequent reactions, an ISPR (in-situ product removal) system needs to be established, with a reactor design shown in figure 5.38-1. By this set up, the zeolite can be easily inserted, removed and the product desorbed. As a control, the same reaction conditions, however without the use of zeolites, were used.

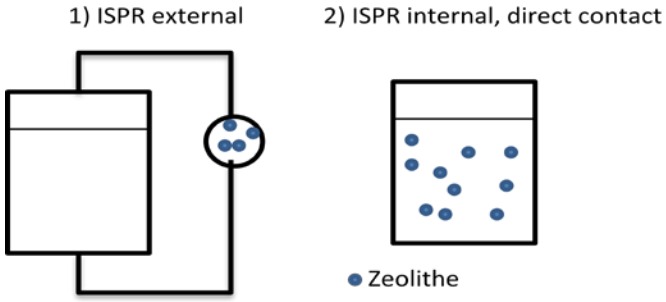


Fig.5.38 Schematics of planned 1) and 2) alternatively used ISPR for preliminary investigations of the trienzymatic system

The reaction was initiated by adding SP, GI granules, and LP into the reaction solution. However, GI granules blocked the filter to the external solid bed adsorber and a circulation of the reaction mixture was not possible anymore. As a result, the zeolites were directly placed into the reaction solution (figure 5.38-2). The trienzymatic reaction did occur and the results are shown in figure 5.39.

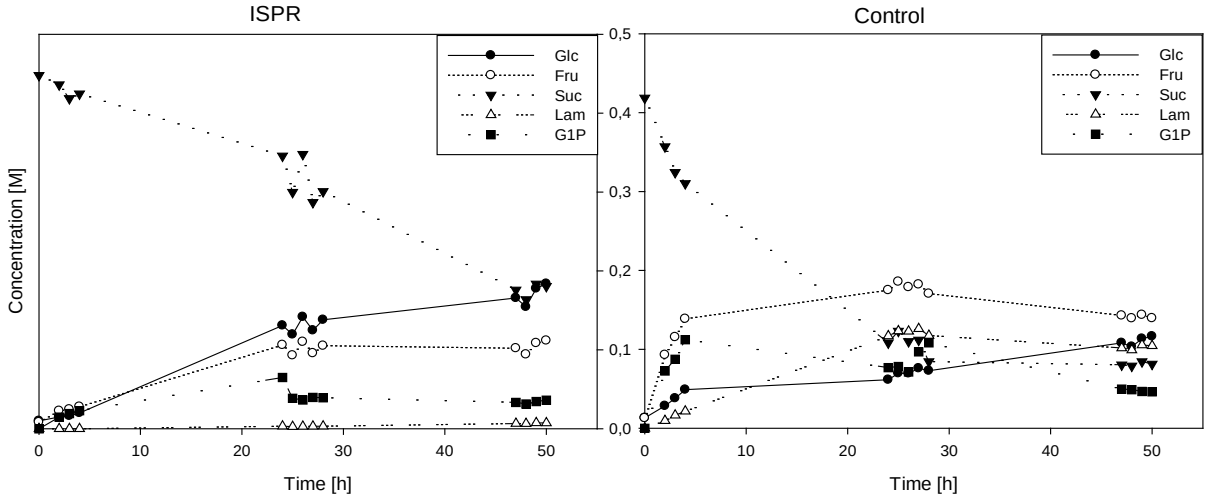


Fig.5.39 Comparison of reaction process of a native trienzymatic system a) ISPR (200 g/l BEA), b) control using 500 mM sucrose in Sorensen buffer (pH 6.2) at 45°C

In the ISPR system with zeolites, the initial pH of 6.2 increased with the reaction duration to an end value of 9.5 after 50 hours. This causes a decrease of SP activity, as suggested by a relatively low conversion rate of sucrose, consequently providing less substrate for GI and LP. The change in pH

favored the activity of GI in the control, as the rate of glucose formation is much higher than for the control. Also there is no evident glucose decrease in the ISPR system, which may suggest an overall low activity for LP due to pH changes.

The observations made indicate that laminaribiose yield is greatly reduced. By comparing the mass balances of laminaribiose and its higher oligomers of ISPR with the control, the concentrations of the control are 0.11 M while the ISPR contains 0.0085 M of laminaribiose. The production of higher oligomers was observed in the control, but nearly no higher oligomers were produced in the presence of zeolite (figure 5.40). Due to the previous statements concerning pH it is likely an adsorption on the zeolite did not occur, as no laminaribiose was produced at all.

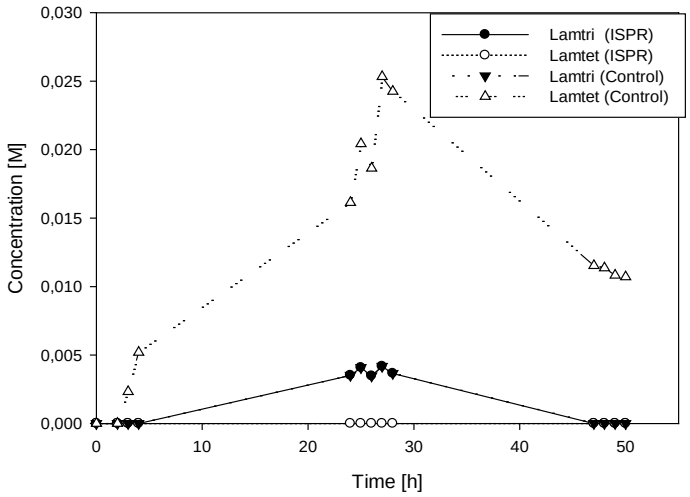


Fig.5.40 Comparison of reaction process of higher oligomers in a trienzymatic system a) ISPR (200 g/l BEA), b) control using 500 mM sucrose in Sorensen buffer (pH 6.2) at 45°C

Over time the GI granules were dissolved in the reaction mixture, making it impossible to separate the zeolites from the solution. Consequently, it is not possible to determine how much of laminaribiose was actually adsorbed and purified. For the following investigations of a native ISPR, native, enzymatic GI is used, resulting in a homogeneous solution in which the zeolites can be obtained by centrifugation.

What is to be taken from the preliminary experiments of the ISPR is that a pH change caused by the zeolite will occur, causing a highly reduced activity of SP and a complete inactivation of LP. The pH of the ISPR needs to be controlled and adjusted, so that the system maintains its initial pH value.

5.7.2 ISPR with different zeolites types using native enzymes

Different zeolites types were compared in order to determine the best choice for the product isolation in an ISPR (table 5.12).

Tab.5.12 Zeolite types used for direct contact ISPR

R1	R2	R3	R4
control	BEA50	BEA150	BEA extrudate

Due to the fact that a desorption of laminaribiose from the zeolite was not carried out, the amount of laminaribiose adsorbed can only be calculated by mass balances. If an adsorption happened, the mass balances at the end of the reaction should be less than at the beginning. The difference in those two balances theoretically represent the amount of laminaribiose adsorbed in the zeolite. The mass balance of all components in the mixture at the beginning and end of the reaction is shown by figure 5.41.

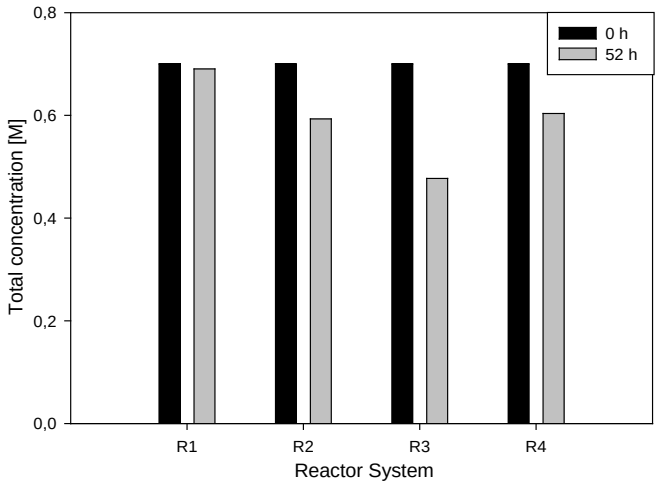


Fig.5.41 Comparison of mass balances at beginning of reaction (0 h) and end of reaction (52 h). Differences between mass balances show theoretical concentration of laminaribiose adsorbed in zeolites, using 700 mM sucrose in Sorensen buffer (pH 6.2) at 45°C

Mass balances for the control (R1) show nearly identical values. The total amount of ß-1.3 oligomers formed were 0.16 M (0.12 M Lam and 0.04 M higher oligomers). The concentrations of laminaribiose theoretically adsorbed in the zeolites are shown in table 5.13.

Tab.5.13 Theoretical concentrations of lam adsorbed on zeolites

R1 [M]	R2 [M]	R3 [M]	R4 [M]
-	0.11	0.22	0.10

In all cases laminaribiose adsorbed on the zeolites. R2 and R4 have nearly identical values with 0.10 M laminaribiose adsorbed. For R2 and R4, 0.08 M and 0.1 M laminaribiose remained unadsorbed inside the reaction solution, with 0.005 M laminaritriose produced in R2. The total amount of higher oligomers produced in R2 and R4 are 0.195 M and 0.2 M respectively, and lie in the same range as the total mass balance of R1 (0.16 M). This suggests that 50% of the produced laminaribiose adsorbed on the zeolites. Interestingly a soaring rise of laminaritriose in R4 was observed after 60 hours reaction. This is most likely caused by an overload of the zeolite extrudates, which block further adsorption.

Mass balances remained constant after 52 hours, suggesting that no adsorption takes place. In addition, higher oligomers accumulated in the reaction solution, further supporting the assumed overload of the zeolite extrudates.

Zeolites used in R3 adsorbed 0.22 M laminaribiose, corresponding to an adsorption of nearly 100 %, since no remaining laminaribiose or higher oligomer concentrations were detected in the reaction solution. Furthermore, 0.22 M lies within the same total mass balance of oligomers as in R1, R2, and R4, so it can be assumed that the theoretical calculations for the mass balances in all systems are correct. The amount of laminaribiose adsorbed in all reactors are significantly higher than expected in the work of Waluga (Waluga 2013, Dissertation). Based on the equilibrium data only 30-40 mM laminaribiose should have been adsorbed on the zeolites. It must be noted, that not only laminaribiose adsorbs on the zeolite, but also an co-adsorption of glucose and fructose as shown by Waluga occurs (Waluga 2013, Dissertation). The model of Markham and Benton should be applied to calculate the concentrations of carbohydrates adsorbed in the zeolites (Waluga 2013; Dissertation). Simplified, at low carbohydrate concentrations, each sugar makes of 1/3 of the total concentration inside the zeolite. When taking this into consideration for 0.1 M of total sugar adsorbed (R2, R4), approximately 30 mM of laminaribiose, glucose, and fructose adsorbed in the zeolite, which would then correspond to the equilibrium data found by Waluga. For 0.2 M of total carbohydrates adsorbed (R3), 67 mM of laminaribiose is theoretically adsorbed in the zeolite, which exceeds the proposed equilibrium data. It is possible that the simplified approach does not fully apply in this case.

Due to the comparison of the mass balances and the theoretical amount of laminaribiose adsorbed in zeolites, it is recommended to use BEA150 for an immobilized trienzymatic reaction with an external solid bed system.

5.7.3 Summary ISPR system

Preliminary experiments of the ISPR showed that a pH change to a more basic value is caused by the zeolite, therefore reducing the activity of SP and completely inactivating LP. The initial pH of the systems needs to be controlled and adjusted if necessary.

In all cases, laminaribiose adsorbed on the zeolites used. For an immobilized trienzymatic reaction BEA150 is recommended, due to highest theoretical adsorption.

5.8 Immobilization of enzymes in chitosan and agar

An economic application of enzymes in a continuous system can only be realized when the enzymes are stable, reusable and production cost are kept low (Buchholz and Kasche 1999). By immobilization it is possible to obtain stable and reusable biocatalysts. In this work two approaches of matrix entrapment techniques were carried out using chitosan and agar.

5.8.1 Chitosan Immobilization

The apparatus used for producing chitosan beads is shown in figure 5.42. The chitosan-acetic acid solution containing the enzyme is placed inside the apparatus and dropped into a polyphosphate solution and stirred for 30-120 minutes.

Horizontal and vertical air pressure allows control of form and size of the beads. In order to reduce mass transport limitations, it is important to keep the bead diameter as small as possible (Baerns 2006). Regarding technical application, a certain diameter however is needed.

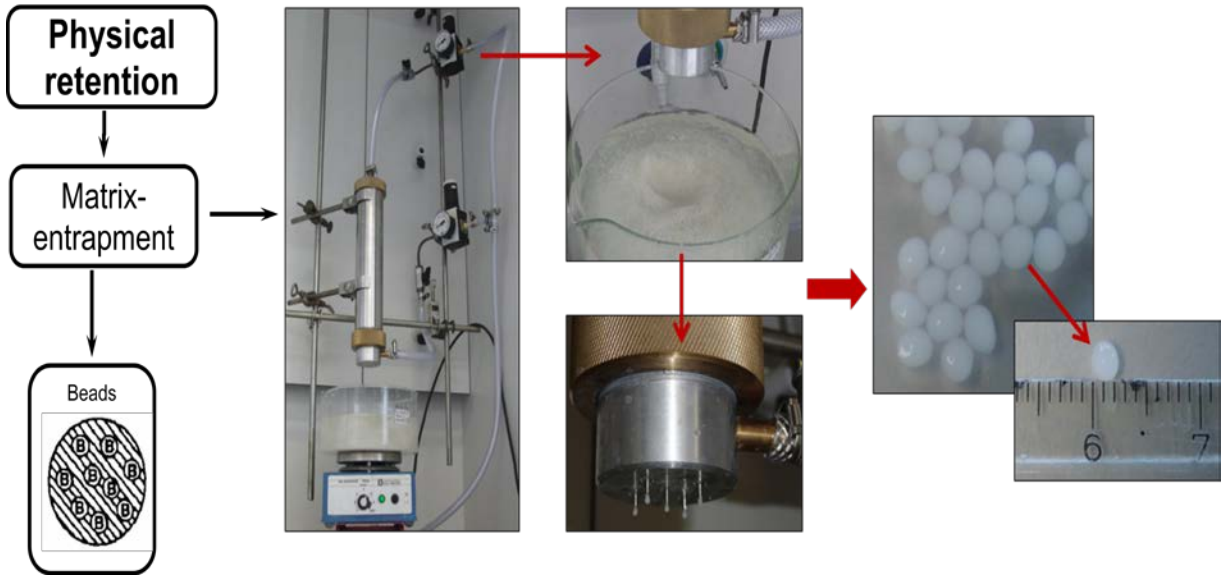
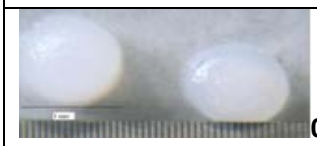


Fig.5.42 Apparatus and equipment for immobilization of the enzymes (from Zwerenz 2012, Dissertation)

5.8.1.1 Beads from pure chitosan solution

In preliminary tests, conditions for getting beads with desired size and form were evaluated. Therefore a pure 1 ml 2% chitosan-acetic acid solution (supplied by Waluga, see also Waluga, Dissertation 2013) was dropped into a 60 ml 10% Na-Polyphosphate solution (pH 5.5) and stirred for 30 minutes. The horizontal and vertical pressures were varied from 0-0.5 bar. Table 5.14 represents the beads after varying the vertical pressure.

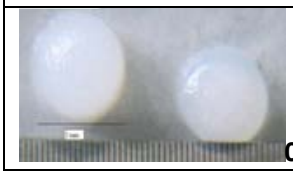
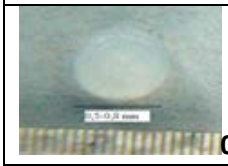
Tab.5.14 Beads created with variation of vertical pressure (0-0.3 bar) after 12 h storage

 **0 bar**	- Ø = 2 mm - no visible deformation or ruptures
 **0.1 bar**	- Ø = 2 mm - no visible deformation or ruptures
 **0.2 bar**	- Ø = 2 mm - ruptures visible
 **0.3 bar**	- Ø = 2 mm - beads burst open

By reducing the vertical pressure, the horizontal pressure will automatically increase, as both systems are directly connected to the same air flow. It is recommended to use a vertical pressure between 0-0.2 bar to obtain beads that are fully intact.

The results of varying the horizontal flow are shown in table 5.15 with corresponding change in vertical pressure.

Tab.5.15 Beads created with variation of horizontal pressure (0-0.3 bar) after 12 h storage

 **0.1 bar**	- Ø = 1 mm - no visible deformation or ruptures - vertical pressure = 0.2 bar
 **0.15 bar**	- Ø = 1 mm - no visible deformation or ruptures - vertical pressure = 0.2 bar
 **0.2 bar**	- Ø = 1 mm - ruptures visible - vertical pressure = 0.2 bar
 **0.3 bar**	- Ø = 0.5-0.8 mm - unstable - vertical pressure = 0.3 bar
 **0 bar (fully closed)**	- Ø = - mm (not measurable) - beads stick together - vertical pressure = 0.5 bar

Relatively uniform and round beads were obtained using horizontal pressures between 0.1-0.2 bar. Though the smallest bead diameter was achieved at 0.3 bar the beads could not be used as they were highly unstable. Most beads dropping onto the surface of the polyphosphate solution instantaneously burst open, resulting in irregular shaped particles with a broad size distribution. Reducing the dropping height to a minimum did not contribute to obtaining uniform beads sizes and forms.

Based on the results, vertical/horizontal pressures of 0.2 bar each were used for further immobilization. The stirring speed of the polyphosphate solution is to be kept relatively low in order not to destroy the beads due to shear stress. Not stirring the solution will cause a clumping of the beads.

5.8.1.2 Immobilization of laminaribiose phosphorylase with chitosan

For the immobilization of laminaribiose phosphorylase, 0.06 U of the enzyme suspension was immobilized in 5 ml 1.5-2% chitosan-10%-15% PP solution. It must be noted, that immobilization was initially carried out with purified and concentrated LP solution, which however was not dialyzed. The enzyme suspension-chitosan mixture dissolved instantaneously when they came in contact with the polyphosphate solution. This may be caused by the ammonium sulfate residues in the LP solution, blocking the formation of the entrapment matrix. Prior to immobilization dialysis has to be performed, in order not only to concentrate LP, but also to eliminate ammonium sulfate residues.

The obtained beads were of similar size and form with a diameter of 1-1.5 mm (figure 5.43). Characteristic is the bright yellow color, caused by LP itself, as the enzyme´s suspension is yellow-orange colored.

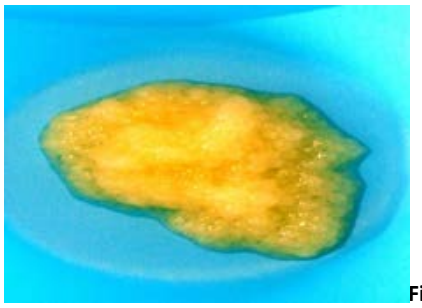


Fig.5.43 LP-chitosan beads after immobilization

Activity tests of the immobilized LPs were carried out (figure 5.44)

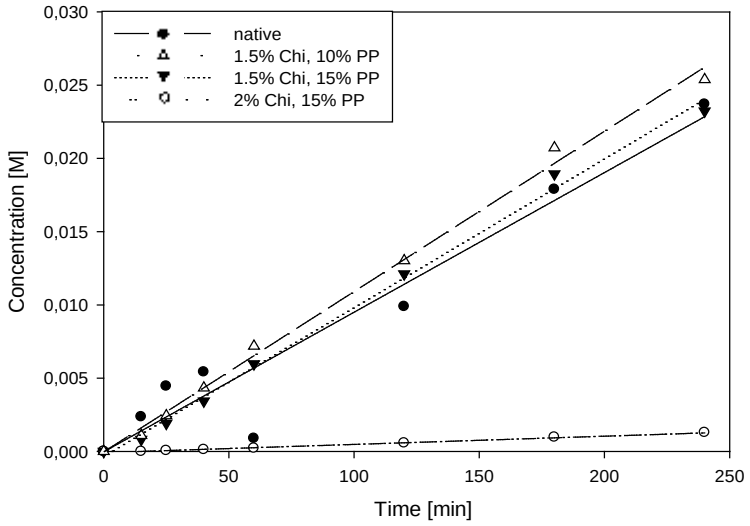


Fig.5.44 Laminaribiose production using 450 µl LP immobilized in 1.5%-10%, 1.5%-15%, 2%-15% chitosan-acetic acid with 0.25 M Glc and 0.15 M G1P at 38°C within 6 hours

The initial reaction rate for both 1.5% Chi in 10-15%PP was 0.1 mM/min with a 20% laminaribiose yield after 240 minutes, in terms of G1P consumption, which corresponds to the initial reaction rate of native LP. Only slight amounts of laminaritriose concentrations were produced, with initial rates of 0.01 mM/min. This also is in accordance with the values obtained for the native enzyme.

For LP immobilized in 2% Chi in 15% PP, only a negligible activity was observed with an initial reaction rate 0.006 mM/min and a laminaribiose yield of 3%, in terms of glucose-1-phosphate consumption, after 24 hours reaction time. It is likely that 2% chitosan-15% PP solution caused a great reduction of the enzyme´s activity.

The kinetics of the immobilized biocatalysts were studied and compared with the native enzyme (table 5.16) by using equation 5.2-5.5. Due to the low activity of LP immobilized in 2% chitosan, the kinetic parameters could not be determined.

Tab.5.16 Modeling of kinetic parameters for LP

Chito [%]	PP [%]	K_{MG1P} [mM]	K_{MGlc} [mM]	K_{MLam} [mM]	v_{maxLam} [mM·min^{-1}]	$v_{maxLamtri}$ [mM·min^{-1}]	R^2 -
native LP		363	3.2	0.007	0.05	0.003	0.99
1.5	10	290	2.7	0.008	0.06	0.004	0.97
1.5	15	429	5.2	0.01	0.02	0.002	0.99

Production of higher oligomers in the form of laminaritetraose and –pentaose was not detected in the observed time range. Modeling results show an improvement of the kinetic parameters when LP is immobilized in 1.5% chitosan and 10% polyphosphate. Affinities for the substrates increased while the maximum reaction rates stayed relatively constant. The matrix may give LP a stabilizing effect, contributing to an increased enzymatic activity. Opposite results were obtained for the immobilization

in 1.5% chitosan and 15 % polyphosphate. K_M values significantly increased, meaning higher substrate concentrations are needed for LP to remain its efficiency. Maximum reaction rates decreased to 0.01 $mM \cdot min^{-1}$ in terms of laminaribiose formation and laminaritriose formation was at 0.002 $mM \cdot min^{-1}$. This can only be explained by the usage of the 15% PP which causes unfavorable conditions for LP.

5.8.1.3 Immobilization of sucrose phosphorylase with chitosan

For the preliminary immobilization of sucrose phosphorylase, 0.3 U enzyme solution was immobilized in 5 ml 2% chitosan-15% PP solution. The obtained beads were of similar size and form with diameters of 1-1.5 mm (figure 5.45), with a characteristic white color.

Fig.5.45 SP-chitosan beads after immobilization

An activity test was carried out using 400 mM sucrose for 2 hours at 38°C (figure 5.46).

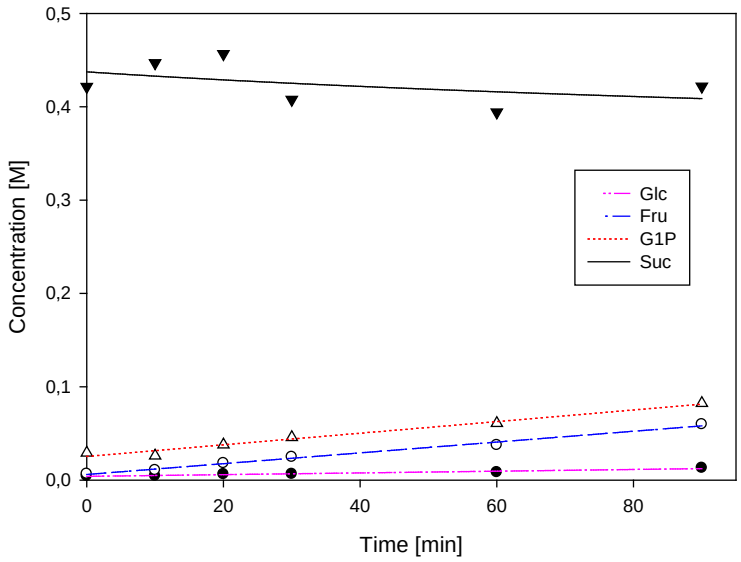


Fig.5.46 Phosphorolysis of 400 mM Suc in Sorensen buffer (pH 7) using SP immobilized in 2% chitosan-acetic acid solution at 38°C within 90 minutes.

Immobilized sucrose phosphorylase showed an initial reaction rate of 0.5 mM/min. This equals to a remaining activity of only 10% in comparison with the native enzyme. Chitosan concentrations were varied, keeping PP concentrations constant, and similar activity losses were found. There was no enzymatic activity detected in the polyphosphate solution, hence no loss of enzyme during the immobilization process itself occurred. However a loss in enzyme concentration will happen, since

small amounts of chitosan, containing SP, will reside in the immobilization apparatus. This is true for the other enzymes as well.

The kinetics of the immobilized biocatalysts within chitosan/polyphosphate was studied and compared with the native enzyme (table 5.17) by using equation 5.11. The model was used on a set of reactions with 6 different sucrose concentrations. For all combinations of embedding agents the v_{Max} value decreased significantly, in comparison with the modeled value for the native enzyme. This is most likely caused by the chitosan-polyphosphate matrix, which blocks the active site of the enzyme. The K_M was found to be relatively constant, with K_{MSuc} at 1.5% Chitosan and 15% polyphosphate being an outlier, implying that the reduced rate cannot be attributed to diffusion limitation.

Tab.5.17 Comparison of kinetic parameters for SP significant for G1P production

Chitosan [%]	Polyphosphate [%]	K_{MSuc} [mM]	v'_{maxSuc} [mM·min^{-1}]	R^2 -
native SP		0.7	30	0.95
1.5	10	0.2	0.4	0.99
1.5	15	10	0.5	0.90
2	15	0.1	0.5	0.99

5.8.1.4 Immobilization of glucose isomerase with chitosan

Before immobilization within chitosan/polyphosphate, matrix GI granules had to be finely grinded in order to avoid the immobilization apparatus being blocked by the granules and to ensure an equal distribution of activity over the whole bead volume. The grinded GI granules had to be checked for their activity with respect to the influence of the mechanical and thermal shear/stress. 50 mg of GI granules were grinded and an activity test carried out, using 400 mM fructose (figure 5.47).

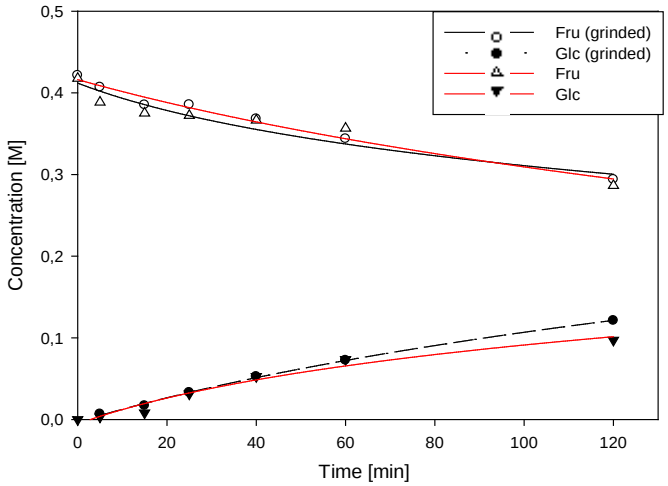


Fig.5.47 Isomerization of 400 mM Fru using grinded and non-grinded GI granules at 45°C

Grinding GI granules does not destroy the enzyme as the activity remains the same with an initial reaction rate of 1.3 mM/min, which corresponds to the rate of the non-grinded GI granules.

25 mg of the grinded GI granules were immobilized in 7 ml 1.5%-2% chitosan-10-15% PP solution. Beads were obtained with diameters of 1.5-2 mm and a characteristic red color. The slight increase of diameter in comparison with SP and LP can be explained by the solid GI content (figure 5.48).

Fig.5.48 GI-chitosan beads after immobilization

An activity test of immobilized GI was performed (figure 5.49), with an initial reaction rate of 1.9 mM/min. This corresponds to an increase of 0.6 mM/min in comparison with the reaction rate of GI granules (1.3 mM/min), caused by the adding of an Mg^{2+} activator. An activity of the polyphosphate solution after immobilization was not observed. A loss of enzyme during the process itself did not occur.

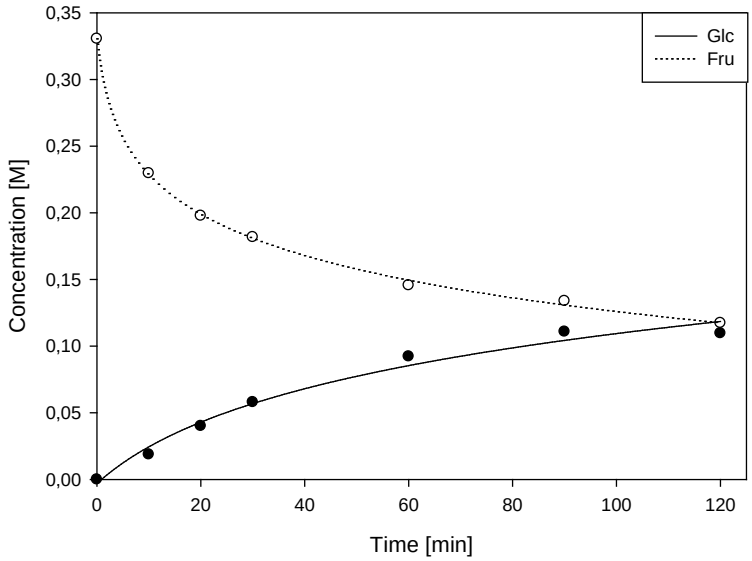


Fig.5.49 Isomerization of 400 mM Fru using grinded GI granules immobilized in 2% chitosan-10% PP at 45°C.

The high decline of fructose concentration immediately after the beginning of the reaction is due to the diffusion of the substrate into the bead. The diffusion coefficients for fructose and glucose can be found in the work of Waluga (Waluga, Dissertation 2013).

Additionally, a GI solution, kindly provided by Novozymes A/S, was also tried to immobilize in a chitosan-polyphosphate matrix. While mixing the enzyme with the chitosan solution it was noticed that a white precipitation occurred, suggesting GI was denaturized during the process. The denaturation was caused by acetic-acid. Contrary to GI granules, native GI is highly sensitive to pH

changes and denatures irreversible in an acidic medium. As a result it is not possible to immobilize native GI in a chitosan matrix, regardless how little the acetic-acid concentration in the chitosan solution.

The kinetic parameters were determined by modeling the experimental data with equation 5.13 on a set of reactions with 6 different fructose concentrations (table 5.18). Overall, K_{MFru} and K_{MGlc} was changed by 35% to 50% depending on the embedding agent concentrations used for immobilization. More importantly v_{max} values did not decrease significantly after immobilization and are comparable with the maximum reaction rates of the GI granules.

Tab.5.18 Comparison of K_M und v_{max} for GI

Chitosan %	Polyphosphate %	K_{Fru} mM	K_{Glu} mM	v_{maxFru} mM·min^{-1}	v_{maxGlc} mM·min^{-1}	R^2
GI Granules		300	360	6.3	2.9	0.98
1.5	10	420	350	3.5	4.0	0.96
1.5	15	260	440	3.1	4.3	0.93
2	15	390	400	5.3	3.4	0.96

5.8.1.5 Catalytic effectiveness

The diffusion coefficients of the participating reactants and intermediates in a chitosan-polyphosphate matrix were determined (table 5.19) (Waluga, Dissertation 2013). In the work of Waluga, diffusion coefficients for both membranes and beads were investigated. Relevant for this thesis are the diffusion coefficients of the beads, which had a diameter of 3-4 mm.

Tab.5.19 Diffusion coefficients for Fru, Glc, Suc, G1P in a chitosan-polyphosphate matrix at room temperature (membrane) and at 25°C (beads) (table taken from Waluga, Dissertation 2013)

Sample (%Chi.-%PP-pH)	D_{Fru} 10^{-10} m^{2}s^{-1} (membrane)	D_{Fru} 10^{-10} m^{2}s^{-1} (bead)	D_{Glc} 10^{-10} m^{2}s^{-1} (membrane)	D_{Glc} 10^{-10} m^{2}s^{-1} (bead)	D_{Suc} 10^{-10} m^{2}s^{-1} (membrane)	D_{G1P} 10^{-10} m^{2}s^{-1} (membrane)
1.5-15-5.5*	2.8-3.8	**	2.7-3.7	**	2.1-2.8	
1.8-15-5.5	2.8 (R^2 0.99)	**	2.5 (R^2 0.99)	**	2.0 (R^2 0.98)	
2-18-5.5	3.0 (R^2 0.99)	1.6 (R^2 0.80)	2.1 (R^2 0.99)	2.0 (R^2 0.87)	1.8 (R^2 0.99)	
2-15-5.5	2.4 (R^2 0.99)	1.9 (R^2 0.83)	2.0 (R^2 0.99)	2.5 (R^2 0.84)	1.7 (R^2 0.99)	2.0 (R^2 0.99)
2-12-5.5	2.4 (R^2 0.99)	2.2 (R^2 0.88)	2.5 (R^2 0.99)	2.8 (R^2 0.86)	1.8 (R^2 0,95)	
2-15-8.5	2.5 (R^2 0.99)	2.2 (R^2 0.77)	2.5 (R^2 0.99)	3.0 (R^2 0.88)	2.0 (R^2 0.99)	
Free diffusion coeff.	7.0 Uedaira 1970		7.0 Berensmeier 2004		5.2	

*Membrane was very soft; membrane thickness was not measurable
**beads were mechanically not stabile

If the kinetic data of the enzymes and the given diffusion coefficients are put in relation, the ϕ values for each of the three enzymes were obtained:

$$\phi_{SP} > 10 \qquad (5.18)$$

$$\phi_{GI} = 0.5 - 1 \qquad (5.19)$$

$$\phi_{LP} < 0.1 \qquad (5.20)$$

Under usage of equation 3.9, catalyst effectiveness (eq. 3.10) for SP, GI, and LP are at 40%, 95%, and 95% respectively.

5.8.2 Agar Immobilization

In order to have another option in terms of matrix immobilization, agar was used as a second technique.

5.8.2.1 Preliminary investigations of agar beads

Agar particles of different concentrations (1.5%-10%) were prepared, cut into cubes and their chemical stability observed for a couple of days at 45°C, matching the operating temperature of the enzymes. Only particles with 1.5% agar dissolved after a few hours. Due to the solidifying point at 30°C-42°C (Koch 1881), preparing agar beads is sophisticated, as they will harden out as soon as they are poured into the immobilization apparatus and therefore blocking the jets. The apparatus could be heated, however this would lead to a denaturation of LP and SP, as a minimum temperature between 45°C-48°C is required in order to minimize a direct solidification of the agar when it comes in contact with the metal of the immobilization apparatus. All reactions were carried out in Sorensen buffer.

5.8.2.2 Immobilization of LP with 4% agar

For immobilization, 0.06 U was immobilized in 4% agar (40 g/l, 5 ml total volume) and cut into approximately evenly shaped cubes (figure 5.50) and an activity test carried out.

Fig.5.50 LP-4% agar cubes after immobilization

The decrease of the initial reaction rate from 15 mM/min (native LP) to 11 mM/min was caused by immobilization (figure 5.51a)), corresponding to a loss in activity of 27%. The initial formation rate of laminaritriose has also been reduced, with a remaining activity of 63% and value of 10 mM/min as opposed to 16 mM/min when using native LP (figure 5.52b).

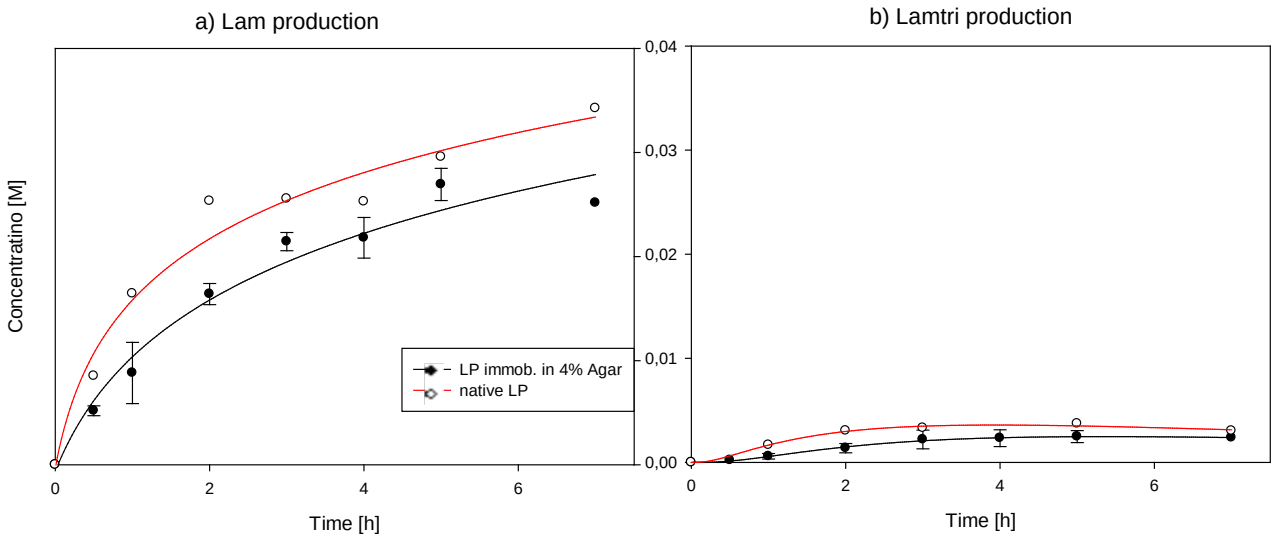


Fig.5.51a/b Comparison of a) laminaribiose and b) laminaritriose formation using nat. and agar immob. LP with 400 mM glucose and G1P in Sorensen buffer (pH 6.2) at 45°C within 8 hours.

The basic two substrate equation was extended with the subsequent reaction to laminaritriose. The model was used on a set of reactions with 5 different G1P concentrations (table 5.20).

Tab.5.20 Comparison of kinetic parameters for LP

	K_{MGlc} [mM]	K_{MG1P} [mM]	K_{MLam} [mM]	v_{maxLam} [mM·min^{-1}]	V_{Lamtri} [mM·min^{-1}]	R^2 -
native LP	363	3.2	0.007	0.05	0.003	0.99
agar LP	102	4	0.012	0.07	0.003	0.94

The kinetic parameters of agar immobilized biocatalyst have increased the LP activity. In terms of the formation of laminaribiose the parameters are more favorable with significantly higher affinity of the reaction with glucose (102 mM). The affinity for laminaribiose as a substrate is reduced, as K_{MLam} is increased by 70% after immobilization. Maximum reaction rates remained relatively unchanged after immobilization.

5.8.2.3 Immobilization of SP with 4 % agar

For the immobilization, SP with an activity of 0.3 U was immobilized in 4% agar (40 g/l, 5 ml total volume) and cut into approximately evenly shaped cubes (figure 5.52).

Fig.5.52 SP- 4% agar cubes after immobilization

An activity test was carried out using 100 mM sucrose in Sorensen buffer (pH 6.2) for 7 hours at 45°C (figure 5.53). Immobilized SP had an initial reaction rate of 5.3 mM/min. This corresponds to remaining activity of 30% in comparison with native SP.

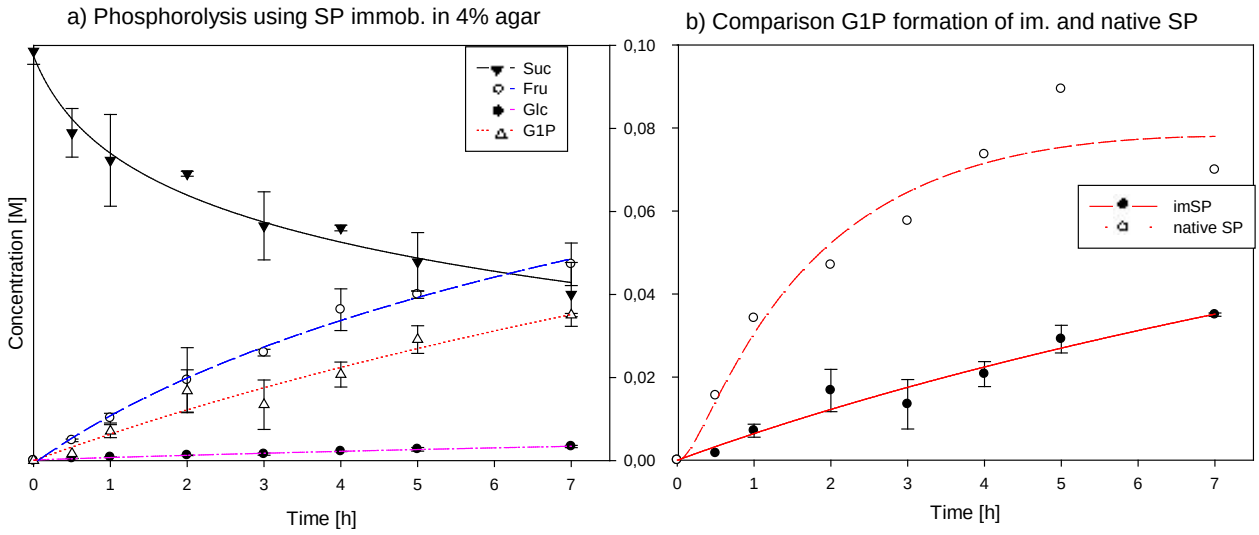


Fig.5.53 a) Phosphorolysis of 100 mM Suc in Sorensen buffer (pH 6.2) using SP immobilized in 4% agar at 45°C within 7 hours; b) comparison of G1P formation with 4% agar immob. SP and native SP

The kinetic model for SP was used on a set of reactions with 6 different sucrose concentrations (table 5.21). V_{Max} of sucrose conversion of agar immobilized SP decreased to half of its native enzyme value, corresponding to 12 $mM \cdot min^{-1}$.This is reasonable when considering that due to mass transfer limitations, the substrate concentration within agar is reduced. The K_M was found to be the same size of magnitude, with K_{MSuc} of 1.3 mM, which however contradicts substance transport limitations. Reasons may be due to non-enclosure of SP in agar and partial denaturation.

Tab.5.21 Comparison of kinetic parameters for SP significant for G1P production

	K_{MSuc} [mM]	v'_{maxSuc} [$mM \cdot min^{-1}$]	R^2 -
native SP	0.7	30	0.95
Agar SP	1.3	12	0.93

5.8.2.4 Immobilization of GI with agar

Due to fixation of GI in the glutaraldehyde crosslinked cells, a bleeding out of the GI will not happen. Therefore only 1.5% agar was used for immobilization. GI was finely grinded and immobilized. However, it was observed that the cubes easily ´broke into many pieces´ already when small pressures or shear stress was applied. This led to a ´muddy solution´ of the reaction mixture, making it very difficult to be adequate for use with an ISPR. The combination of agar and grinded granules lead to very hard and stiff cubes, which will easily break when stirred, rotated, pressure applied etc. This effect increases with increasing agar concentration.

In order to circumvent this issue, the immobilization in 4% agar was carried out using non-purchasable native, enzymatic solution of GI, provided by Novozymes (figure 5.54).

Fig.5.54 Native GI solution-4% agar cubes after immobilization

An activity test was carried out using 800 mM fructose in Sorensen buffer (pH 6.2) for 7 hours at 45°C. It turned out that GI was bleeding out of the agar cubes, due to the fact that no activity of the GI suspension immobilized in agar was measured, while the supernatant showed an enzymatic activity.

In order to increase the matrix´ density, so that GI cannot bleed out anymore, 6% agar (60g/l) was used to immobilize the enzymatic GI solution. The remaining activity lies at 55% compared with the native enzyme (figure 5.55). No activity on the supernatant was observed. The initial reaction rate of immobilized GI had a value of 7.4 mM/min, while the native, enzymatic solution of GI lies at 13.5 mm/min.

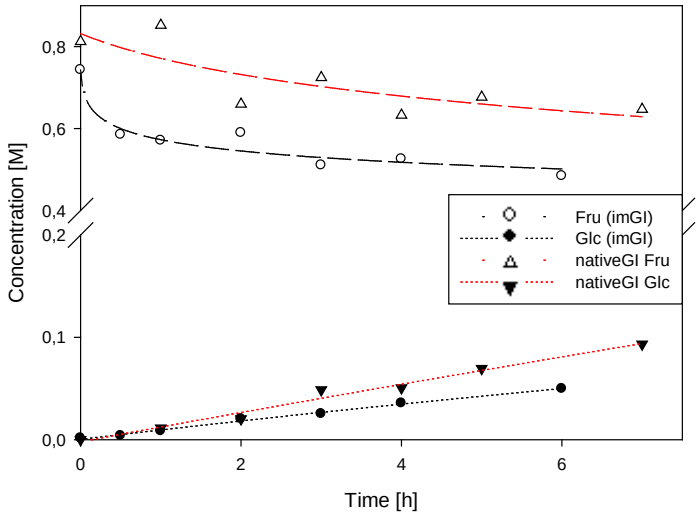


Fig.5.55 Comparison of reaction progress of 0.8 M fructose isomerization in Sorensen buffer (pH 6.2) at 45°C with immobilized native, enzymatic GI in 6% agar and native, enzymatic solution of GI within 7 hours.

Based on the activity tests, kinetic parameters were determined by modeling of the experimental data with equation 5.13. The model was used on a set of reactions with 5 different fructose concentrations (table 5.22).

Tab.5.22 Comparison of K_M und v_{max} for enzymatic GI suspension and immobilized in 6% agar

	K_{Fru} mM	K_{Glu} mM	v_{maxFru} mM·min^{-1}	v_{maxGlc} mM·min^{-1}	R^2
native GI	457	400	1.5	2.0	0.95
agar GI	485	540	1.9	2.0	0.85

Interestingly, immobilization of native GI in agar did not significantly reduce the kinetic parameters. A decrease in the enzymes affinity was observed, due to diffusion barriers. An increase of the maximum reaction rate of fructose conversion was observed. It seems that fructose is converted at a faster pace than glucose is being produced. However fructose must first diffuse into the matrix, before it can actually be converted. Samples were only taken in the reaction mixture itself. As a result it seems that the fructose concentration decreases faster, but may actually have the same rate as the native system. This also provides an explanation for the relatively low regression factor of 0.85.

5.8.3 Comparison of enzymes immobilized in chitosan and agar

Table 5.23 summarizes the kinetic parameters of chitosan and agar immobilized enzymes, obtained with the developed reaction models.

Tab.5.23 Comparison of kinetic parameters of chitosan and agar immobilized enzymes

	K_{MSuc}	K_{MFru}	K_{MGlc} mM	K_{MG1P}	K_{MLam}	v_{maxLam}	V_{Lamtri}	v'_{maxSuc} mM·min^{-1}	v_{maxFru}	v_{maxGlc}	R^2 -
Agar LP			102	4	0.012	0.07	0.003				0.94
Chitosan LP			290	2.7	0.008	0.06	0.004				0.97
Agar SP	1.3							12			0.93
Chitosan SP	0.2							0.4			0.99
Agar GI		485	540						1.9	2.0	0.85
Chitosan GI		260	440						5.3	3.4	0.96

LP immobilized in agar was compared with the 1.5% Chito-10% PP immobilized enzyme, representing the best obtained values. The maximum reaction rates in terms of laminaribiose and -triose formation are practically identical for both immobilization techniques. Agar seems to cause a greater stabilizing effect on LP than chitosan. LP in agar has a much higher affinity towards the reaction with glucose (102 mM) than in chitosan (290 mM). In addition, K_{MLam} for chitosan LP is significantly lower than for agar LP, meaning less concentrations are required to promote the undesired side reaction to laminaritriose. This concludes that agar is the preferred choice of immobilization for LP.

SP immobilized in agar is compared with 1.5% Chito-10% PP immobilized SP. It is to be noticed that the initial reaction rate of SP immobilized in chitosan is at 0.5 mM/min and corresponds to an improvement of residual activity in agar of over 1000 %, making it obvious that SP immobilized in 4% agar seems way more suitable than using chitosan-polyphosphate as an immobilization technique. The maximum reaction rate of agar SP is 12 mM·min^{-1} as opposed to 0.4 mM·min^{-1} of chitosan SP. Evidently, activity loss in agar is significantly less than in chitosan and should be preferred.

GI immobilized in agar and chitosan (1.5% Chito-15% PP) cannot be compared, due to the fact of GI granules used for chitosan immobilization and native GI solution for agar immobilization. From investigation of the native enzymes granule GI has a higher activity than GI suspension, due to stabilizing effects of the glutaraldehyde matrix (Jorgensen et al. 1988). This still applies for the immobilized GI granules, having approximately an overall 50% higher activity than GI suspension.

Chitosan immobilization can be realized at room temperature and hence giving milder immobilization conditions, especially for temperature sensitive enzymes, while immobilization of enzymes in agar at higher temperatures makes the handling of enzymes and their immobilization more difficult. Nonetheless, based on the results, it can be concluded that agar provides a better technique of immobilization. As the immobilized trienzymatic reaction is to be carried out at 45°C, this should also be the maximum temperature used for immobilization of the enzymes in agar.

5.8.4 Summary of enzyme immobilization

Homogeneous and reproducible chitosan-polyphosphate beads with diameters between 1-2 mm were successfully produced by using the immobilization apparatus (figure 5.42). This set-up was used for the immobilization of the enzymes in chitosan and Na-polyphosphate (pH 5.5).

LP immobilized in 2% chitosan-acetic acid solution did not show any activity, probably caused by the inactivation of the LP due to the acetic acid. Hence the acetic acid concentration was reduced and by use of 1.5% chitosan-acetic acid a remaining activity of 0.01 mM/min in terms of laminaribiose production was observed. This rate corresponds to that of the native enzyme. The diameter of the beads ranged between 1-1.5 mm. Maximum reaction rates after immobilization deteriorated greatly in 15% Chi-15% PP. However, a greater activity was obtained when LP is immobilized in 1.5% chitosan and 10% polyphosphate than for native LP, implying a stabilizing effect of the matrix on the enzyme.

The diameter of immobilized SP beads was between 1-1.5 mm. No detectable loss of enzyme was detected. By immobilization, activity of SP was greatly decreased with an initial reaction rate of 0.5 mM/min, corresponding to a reduction in enzyme activity of 90%. This was confirmed by the kinetic parameters, especially for v_{Max} which decreased significantly in comparison with v_{Max} of the native enzyme.

GI granules must be grinded in order to ensure an equal distribution over the whole bead volume and to minimize the risk of the apparatus being blocked by the granules. Immobilized grinded GI granules had an increased diameter of 1.2-2 mm. Grinding GI granules does not affect the enzyme as the activity remains the same as for non-grinded GI granules. Activity tests in the polyphosphate solution did not show a reaction, implying that there no remains of GI granules in the PP solution. The kinetics

parameters showed generally reduced activity. Native GI could not be immobilized in chitosan-polyphosphate as native GI is pH sensitive and denaturizes at low pH (Jorgensen et al. 1988).

To determine the catalytic effectiveness of each enzyme immobilized in chitosan, the Thiele-modulus was calculated. The kinetic data of the enzymes and the given diffusion coefficients, determined by Waluga (Waluga 2013, Dissertation), the catalytic effectiveness for SP, GI, and LP are at 40%, 95%, and 95% respectively.

Preliminary experiments confirmed the applicability of agar as an immobilization matrix. When using 4%-10% agar, the cubes are relatively stable for a long period of time and very resistant in terms of shear stress. For the immobilization 4% agar was applied. The remaining activity of LP was 65% in terms of initial laminaribiose formation rate. Maximum reaction rates remained unchanged and affinity for laminaribiose as a substrate was reduced. SP had a remaining activity of 30%, compared with the native enzyme. Compared with chitosan immobilization, the agar immobilization showed an improvement of residual activity of over 1000%.

For GI, granules cannot be used for immobilization in agar as they will lead to very hard and stiff cubes, making them difficult to handle. The cubes are very sensitive to shear stress, causing the cubes to be destroyed and turning the reaction solution very muddy, hence making it very difficult to be adequate for use with an ISPR. Alternatively, a soluble GI solution was used for immobilization. The use of 6% agar keeps the enzyme inside the cubes and an activity of 55% remained. Kinetic parameters were not significantly reduced after immobilization in agar.

Comparing the two immobilization techniques, revealed a higher enzymatic activity was obtained with agar immobilization and is therefore be favored for establishing an immobilized trienzymatic/ISPR system.

5.9 Immobilized trienzymatic batch reaction

5.9.1 Chitosan immobilized trienzymatic batch reaction

It was shown that agar is more suitable than chitosan for immobilization. Nonetheless a trienzymatic batch reaction with chitosan immobilized enzymes was carried out as kinetic parameters determined after chitosan immobilization are still satisfactory, especially in terms of immobilizing the enzymes in milder conditions (see also 5.8.3).

GI, SP, and LP were separately immobilized in 1.5-2% chitosan-acetic acid solutions. Activity tests were performed using 500 mM sucrose in Sorensen buffer (pH 6.2) at a temperature of 45°C for 24 hours in a batch reactor with a final reaction volume of 30 ml.

No significant production of laminaribiose was observed. Since polyphosphate acts as complex builder for magnesium, resulting in an activity loss of GI during the immobilization process, Mg^{2+} was added as an activator into the reaction solution (Zittan et al. 1975). However this did not affect the process. A white precipitate was formed, most likely caused by the $Mg_3(PO_4)_2$ (from the Sorensen buffer). Adding other suitable ions (Co^{2+}, Mn^{2+}, Zn^{2+}) also resulted in a salt formation blocking the trienzymatic reaction. Furthermore the system was also studied with only immobilized LP and SP and separate addition of GI granules. Activity of GI could be retained and the production of the intermediates was observed (figure 5.56).

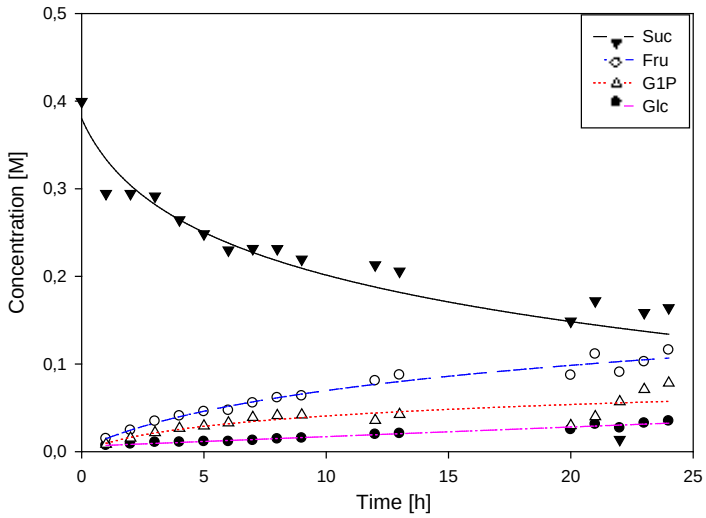


Fig.5.56 Trienzymatic reaction using immobilized SP/LP and GI granules. Final reaction volume: 30 ml: 500 mM Suc in Sorensen buffer (pH 6.2), temperature 45°C, and 24 hours reaction time.

Based on the concentrations of the intermediates required for laminaribiose production and the mass balances, there should be a laminaribiose yield of theoretically 0.05 mM after 24 hours reaction time. So the limiting factor seems to be immobilized LP. It is known that EDTA serves as an activator for LP (Manners and Taylor 1967) and was added to the reaction mixture (figure 5.57).

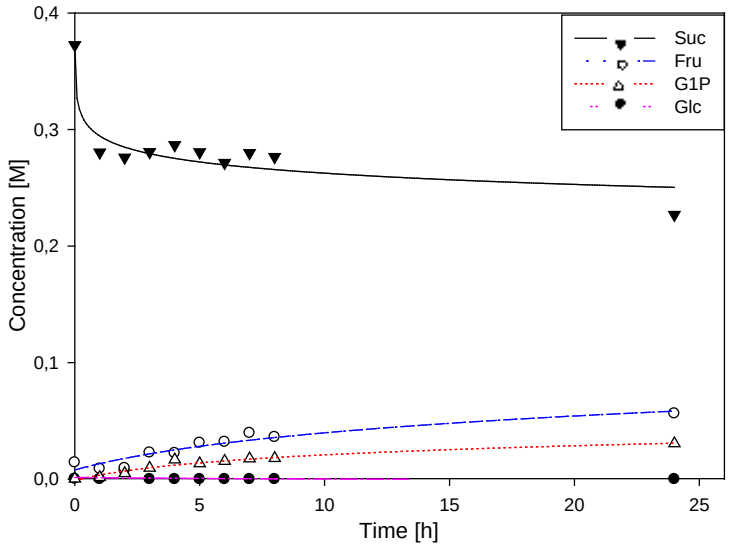


Fig.5.57 Trienzymatic reaction using immobilized SP/LP and GI granules. Final reaction volume 30 ml, 500 mM Suc in Sorensen buffer (pH 6.2), 2 mM EDTA, temperature 45°C, and 24 hours reaction time

Isomerization of fructose did not occur, due to the formation of a chelate complex from Mg-Ions and EDTA (GESTIS-Stoffdatenbank, IFA). Furthermore, EDTA seems also to inhibit SP, since only small amounts of G1P were obtained.

It must be concluded that the establishment of an immobilized trienzymatic system using chitosan/polyphosphate as an entrapment matrix cannot be used, since the activity loss of the combined immobilized enzymes is too high and no acceptable reaction rates are obtained.

5.9.2 Agar immobilized trienzymatic batch reaction

Based on the results of the enzymes being immobilized in agar, an activity test was performed using 500 mM sucrose in Sorensen buffer (pH 6.2) at a temperature of 45°C for in a batch reactor with a final reaction volume of 60 ml.

Compared to immobilization in chitosan a higher activity in terms of intermediate production was obtained with agar immobilized enzymes (figure 5.58).

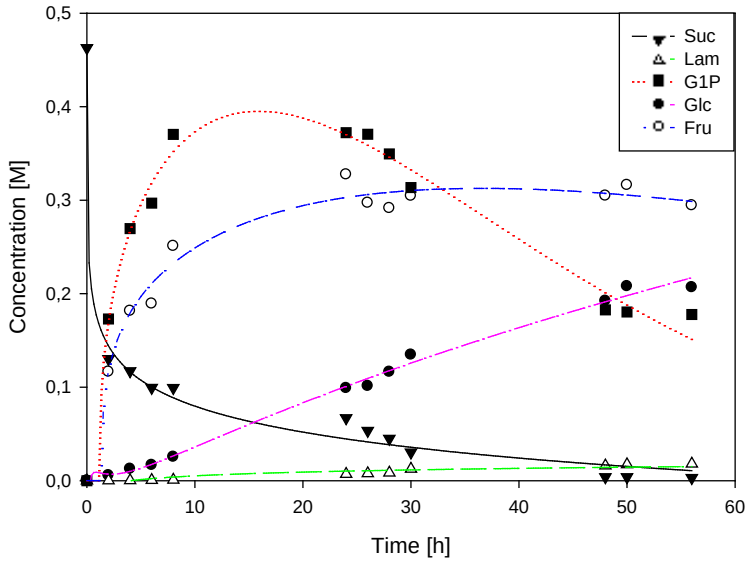


Fig.5.58 Trienzymatic reaction using agar immobilized SP/LP/GI. Final reaction volume: 60 ml, 500 mM Suc in Sorensen buffer (pH 6.2), temperature 45°C, and 72 hours reaction time

It can be concluded that there is nearly a 90% yield of glucose-1-phosphate. The quick drop of sucrose is explained by the diffusion into the agar cubes. Also GI remained active during the entire process. The increase of the concentrations, however also suggests a highly reduced activity of LP, as in the native system glucose concentrations remained at a very low level during the reaction.

Nonetheless, laminaribiose was produced in the batch immobilized trienzymatic reaction system with no side reactions to higher oligomers. Although the yields of laminaribiose obtained are relatively low at 5%, based on sucrose consumption, it is evident that agar immobilization is promising for the establishment of an immobilized trienzymatic system. Obviously, methods must be found to optimize the laminaribiose yields which could not be further investigated in this thesis due to time limits.

5.9.3 Summary of immobilized trienzymatic system

For the trienzymatic system to be used on a larger reactor scale, it must be established with the required enzymes immobilized. Results showed that a chitosan immobilized system cannot be established, due to significant activity loss of LP combined in one operating reactor. No production of laminaribiose was observed.

Contrary to chitosan, a trienzymatic system with the enzymes immobilized in agar showed a production of laminaribiose.

6. Summary

Carbohydrates are essential elements in daily nutrition. Due to their prebiotic effects, they have become of great interest for the growing market of ´functional food´ as well as for the chemical and pharmaceutical industry (Buchholz and Seibel 2003). An especially interesting type of ß-1.3-linked glycosides is laminaribiose.

In this thesis, sucrose is used as a substrate with the three enzymes sucrose phosphorylase (SP), glucose isomerase (GI), and laminaribiose phosphorylase (LP) for the production of laminaribiose by establishing a one-pot process. This trienzymatic reaction serves not only as a source for laminaribiose synthesis but also as a model system for multienzymatic processes. To allow utilization of this system on an industrial scale, immobilization with matrix entrapment using chitosan and agar was studied. If proven successful, an integrated system with the combination of the immobilized biocatalysts needs to be defined and optimized.

To be able to produce laminaribiose from glucose-1-phosphate and glucose, laminaribiose phosphorylase is required. LP must be cultivated and optimized using *Euglena gracilis*. Long term maintanance of *E. gracilis* cultures can be performed for at least three months without contamination or changes in the cultures. A heterotrophic cultivation in the dark for reactivation of a phototrophic culture on agar plates is possible within 7 days. This heterotrophic cultivation was optimized in terms of pH and temperature compared to Kitaoka´s method (Kitaoka et al. 1993). Results show that by keeping the precultures at pH 4.1, 33°C, using citric acid-phosphate buffer (pH 2.6), the lag phase and hence the cultivation time was reduced. Therefore the biomass can be harvested at the beginning of the stationary phase after 96 hours. For an effective cultivation, vitamins B12 and B1 are necessary. With respect to vitamin B12 10 µg/L as suggested in literature is sufficient (Kitaoka et al. 1993). Addition of vitamin B1 for cultivation is not necessary when yeast extract is used as complex nutrient component.

To acquire the LP enzymatic suspension, it is best obtained by carrying out ultrasonic disintegration. For purification of the suspension, ammonium sulfate precipitation was applied. A saturation between 30%-60% contains the fraction with laminaribiose phosphorylase. The purified LP solution exists as a monomer and dimer with molecular masses of approximately 120 kDa and 250 kDa respectively. The production of laminaribiose and the side reactions to higher oligomers in form of laminaritriose and –tetraose were confirmed by activity tests (Kitaoka et al. 1991). Though not determined due to detection limits, hexaoses and heptaoses are also likely to be produced. Optimal working conditions were found between 42°C-45°C at pH 6.2, which is in accordance with the optimum of pH found in literature (Goldemberg et al 1966).

A reaction model for LP kinetics was proposed, enabling the determination of its kinetic parameters. A double substrate mechanism was used as a basis (Goldemberg et al. 1966) and extended to include the side reactions to higher oligomers. Modeling results show a faster laminaribiose formation than oligomer production, which is however highly dependent on the Glc/G1P ratios. Ratios greater than 2 shift the products towards laminaribiose, while greatly reducing higher oligomer formation.

To produce one of the intermediates (glucose-1-phosphate) for laminaribiose phosphorylase, sucrose phosphorylase (SP) was used. SP catalyzes the conversion of sucrose and inorganic phosphate to glucose-1-phosphate and fructose. SP was found to have optimal reaction conditions at 45°C-50°C at pH 7.0. The ping-pong mechanism as shown by Goedl et al. (2008), and used as a basis for modeling, was extended with a hydrolysis term, as glucose-1-phosphate is also a substrate for SP. This leads to a competitive inhibition between sucrose and G1P. Modeling results indicate that phosphorolysis occurs at such high rates, that the hydrolysis rate can be neglected. Low substrate concentrations suggest a shift from product formation to hydrolysis.

The second intermediate for LP to produce laminaribiose is glucose, obtained by the isomerization of fructose by glucose isomerase (GI). With regards to the temperatures used for the other enzymes, the temperature optimum 60°C-80°C for GI as stated in literature (Jorgenson et al. 1988) were not applied in this work. Instead 45°C were used, which is the preferred temperature for operating the trienzymatic system. A reversible mechanism model was applied for the reaction modeling (Converti and Del Borghi 1998).

A bienzymatic system of SP and GI was established in order to study the production of intermediates from sucrose and inorganic phosphate. Results indicate an ´optimum temperature´ in which both enzymes are active is found at 45°C. An inhibition of SP is caused by glucose (Doudoroff 1943), resulting in slightly decreased G1P concentrations. It is important to keep glucose concentrations low, which should be realized when LP is added to the reaction system. This inhibition has to be taken into consideration for modeling the bienzymatic reaction. The concentration gradients of substrates and intermediate products for LP fit very well to the model.

When all enzymes were combined in a trienzymatic system with sucrose and Sorensen buffer, they retained their activities, eventually producing laminaribiose. By comparing the native trienzymatic system to the work of Kitaoka, an increase of laminaribiose yield by 25% was obtained (Kitaoka et al. 1993b). Laminaribiose formation was surpassed by subsequent reactions which lead to the formation laminaritriose and laminaritetraose. This makes it obvious, that laminaribiose needs to be removed from the reaction mixture, via an ISPR (in situ product removal) system. Optimal conditions for the formation of laminaribiose were determined to be at a temperature of 45°C and a pH of 6.2. The

trienzymatic system is well described by combination of the mono-/bienzymatic kinetics, showing a similar trend as the experimental data. However, more experiments will be necessary for more accurate results.

To obtain purified products and minimize the subsequent reaction, an ISPR system needs to be established. It was shown that a pH change to a more basic value is caused by the zeolite, therefore reducing the activity of SP and completely inactivating LP, making a pH adjustment necessary. Different zeolites types have been added into a reaction mixture to determine the best suitable zeolites for the establishment for an ISPR system. In all cases, laminaribiose was adsorbed, with BEA 150 recommended for a trienzymatic reaction due to highest theoretical adsorption.

The establishment of a continuous trienzymatic reaction system requires the immobilization of sucrose phosphorylase, glucose isomerase, and laminaribiose phosphorylase. In this thesis, immobilization in chitosan and agar was investigated. Homogeneous and reproducible chitosan-polyphosphate beads with diameters between 1-2 mm were successfully produced by using the immobilization apparatus (figure 5.42).

Due to inactivation of LP during chitosan-polyphosphate immobilization, probably caused by acetic acid, acetic acid concentration was reduced. A remaining activity of 0.01 mM/min in terms of laminaribiose production was observed, corresponding to the native enzyme. An improvement of enzymatic activity was obtained by immobilization in 1.5% Chitosan and 10% PP, implying a stabilizing effect of the matrix on LP.

Immobilized SP chitosan-polyphosphates beads were produced with no detectable loss of enzyme. A reaction rate of 0.5 mM/min was obtained, corresponding to a reduction in enzyme activity of 90%. The kinetic parameters revealed a great activity loss of SP , especially in terms of v_{Max}, possibly due to the blocking of the active site caused by the immobilization matrix.

For GI to be immobilized in a chitosan-polyphosphate matrix, it must be grinded in order to ensure an equal distribution over the whole bead volume and to minimize the risk of the apparatus being blocked by the granules. Grinded GI granules have the same activity as non-grinded enzymes. The kinetics parameters showed a generally reduced activity. As native GI is denaturized by low pH (Jorgensen et al. 1988), it cannot be immobilized in chitosan-polyphosphate.

With the kinetic data of the enzymes and the given diffusion coefficients (Waluga 2013, Dissertation), the catalytic effectiveness for SP, GI, and LP are at 40%, 95%, and 95% respectively.

Applicability of agar as an immobilization matrix was confirmed by preliminary experiments. Cubes, with 4%-10% agar, are relatively stable and resistant in terms of shear stress. 4% agar was most

suitable for immobilization of the enzymes. For LP, maximum reaction rates remained unchanged and affinity for laminaribiose as a substrate was reduced. Agar immobilization showed an improvement of residual activity of over 1000%, compared to chitosan-polyphosphate immobilization for SP. Agar cubes containing GI granules are very sensitive to shear stress. The causes the cubes to be destroyed and turning the reaction solution very muddy and therefore not suitable for an ISPR system. Instead, a soluble GI solution was immobilized in 4% agar, which however bled out of the cubes. The enzymes were kept inside the cubes by use of 6% agar cubes. Kinetic parameters were not significantly reduced after immobilization in agar.

Comparison of the two immobilization techniques showed that chitosan immobilized enzymes had a lower enzymatic activity than their agar counterparts and is hence to be favored for establishing an immobilized trienzymatic/ISPR system. A trienzymatic system with the enzymes immobilized in agar showed a production of laminaribiose.

7. Outlook

In this work, it was shown that laminaribiose can be produced from an immobilized trienzymatic system and further reducing the formation of higher oligomers by using ISPR. Further investigations could provide more detailed knowledge of the processes involved and therefore optimizing the entire system.

Laminaribiose phosphorylase activity from *E. gracilis* possesses a low activity. New research confirmed the isolation of a bacterial LP from cell-free extract from *Paenibacillus sp. YM-1* (Kitaoka et al. 2012). This could open an interesting approach for the optimization of the enzymatic activity of LP.

To keep the production costs low, the immobilized biocatalyst should be stable, storable, and reusable. Investigations in terms half-life of the enzymatic activity of the immobilisates in the process and their long-term storage are needed.

As seen from the results, a one-pot reactor containing enzymes immobilized in agar lead to the production of laminaribiose. However the yields obtained were relatively low. To compensate the activity loss further research should be performed. This would include optimization of agar immobilization matrix or screenings of other immobilization procedures. Also establishing systems in which the enzymes are placed separately from each other, could contribute to an improvement of the immobilized trienzymatic process. Reactor options could include ´two-pot reactors´ and a cascade system. As seen before, the separate immobilized monoenzymatic systems each contained a relatively high activity and deteriorated once combined in one solution. Merely pumping the mixture through the ´enzymatic compartments´ would allow the enzymes to be separately placed in a closed environment, adjusted to their optimal activity conditions, such as temperature optimum and pH.

A fluidized bed reactor with an external adsorber needs to be established, which contains zeolites packed to a solid bed. The kinetics of the entire process should be determined. The solid bed adsorber offers the possibility for a separate adjustment of optimal design parameters, such as temperature for reaction and adsorption. The results from lab-scale reactors may be transferred to pilot reactors, to gain knowledge of the entire process under realistic conditions. The operation of the pilot technical system was successfully established for the monoenzymatic production of isomaltose (Ergezinger et al. 2006).

A combination of these strategies and detailed analysis may lead to an improved production of laminaribiose and efficient product removal from the reaction mixture. This may prove to be an applicable method of producing laminaribiose and for the process to serve as a model reactor for multienzymatic reactions.

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9. Abbreviations/Symbols

Abbreviations

Abbreviation	Meaning
AHRQ	Agency for Healthcare Research and Quality
approx.	approximately
APS	ammonium peroxo disulfate
Asp	aspartate
BEA	beta polymorph A-type
Bi/Bienzym.	bienzymatic
BSA	bovine serum albumin
Chito	chitosan
conc.	concentration
CORDIS	Community Research and Development Information Service)
DNA/RNA	(deoxy)ribonucleotidic acid
E. gracilis	*Euglena gracilis*
EC	Enzyme commission of IUPAC (International Union of Pure and Applied Chemistry)and IUBMB (International Union of Biochemistry and Molecular Biology)
EDTA	ethylene ditetra amin acetic acid
FAO/WHO	Food and Agriculture organization of the U.N./World Health Organization
Fru	fructose
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
GESTIS	Gefahrstoffinformationssystem
GI	glucose isomerase
Glc	glucose
Glu	glutamate
HFCS	high fructose corn syrup
His	histidine
HPLC/HPAEC	high pressure liquid (anion exchange) chromatography
ICTV	Institut für chemische und thermische Verfahrenstechnik
IFA	Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung
immob.	immobilized
inh.	inhibition

ISPR	in-situ product removal
L. mesenteroides	*Leuconostoc mesenteroides*
Lam	laminaribiose
Lampent	laminaripentaose
Lamtet	laminaritetraose
Lamtri	laminaritriose
LP	laminaribiose phosphorylase
Lys	lysin
MM	Michaelis-Menten
Mono/Monoenzym.	monoenzymatic
NaAc	sodium acetate
nat.	native
OD	optical density
P	inorganic phosphate
PAD	pulsed amperometric detection
PP	polyphosphate
rpm	round per minute
S. Murinus	*Streptomyces murinus*
SAG	Culture Collection of Algae at Göttingen University
SDS	sodium dodecylsulfate
SDS-Page	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	sucrose phosphorylase
Suc	sucrose
Susp.	suspended
TC	thin layer chromatography
TEMED	N,N,N´,N´-tetramethylenethylendiamin
Tri/Trienzym.	trienzymatic
Tris	tris-(hydroxymethyl)-amino ethane
Trp	tryptophane
UDP-	uridinediphosphate
USDA	U.S. Department of Agriculture

Symbols

Symbol	Unit	Meaning
c	[g/l]	concentration
D	[m^2/s]	diffusion coefficient
D_{eff}	[m^2/s]	effective diffusion coefficient
Ea	[kJ]	activation energy
i	[mA]	current
K_L	[$g_{adsorbed}/g_{carrier}$]	Langmuir coefficient
K_m	[M]	Michaelis constant
k_x	[M/s]	reaction rate constant
l	[nm-cm]	length
M	[mol/l]	Molarity
m	[µg-kg]	mass
n	[mol]	mole
R	[8.314 J/K·mol]	universal gas constant
r_0	[m]	diffusion distance, pellet radius
t	[s-h]	time
T	[°C]	temperature
U	[µmol/min]	enzyme activity
$U/g_{protein}$	[µmol/min/g]	specific enzyme activity
V	[µl-l]	volume
v	[V]	voltage
v_{max}	[M/min]	max. reaction rate
v_x	[M/min]	reaction rate
W	[W]	output

Greek letters

Letter	Unit	Meaning
ε	-	porosity
ϕ	-	Thiele modulus
η	-	system effectiveness
λ	[nm]	wave length
τ	-	tortuosity

10. Figures/Tables

Figures

Tables

11. Appendix

11.1 Model diagrams of LP kinetics using ModelMaker3®

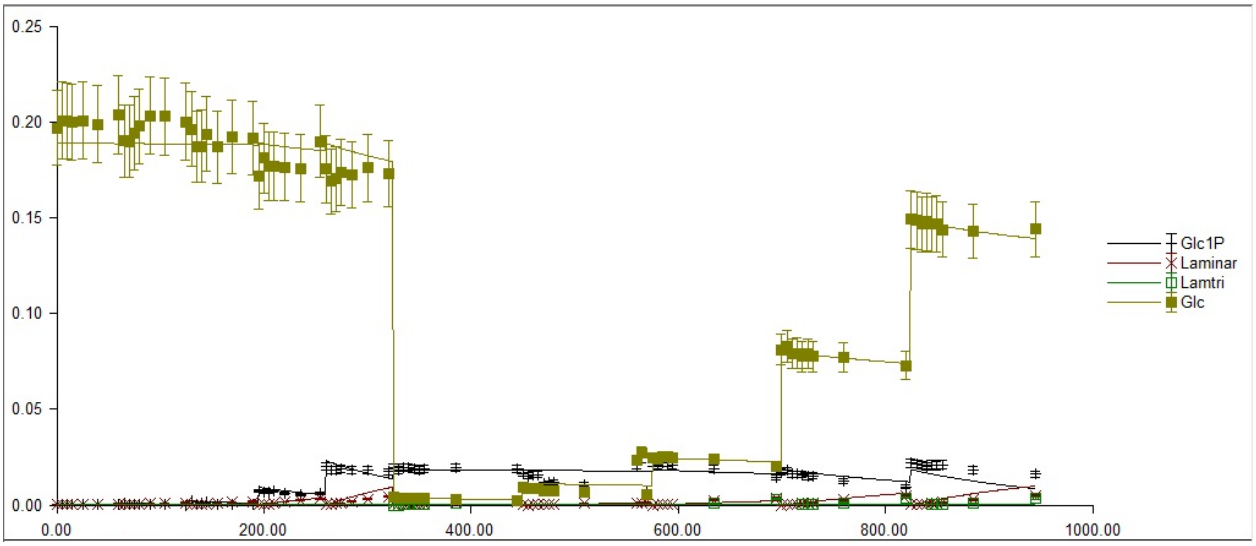

Fig. 11.1 MM3 Models of native LP kinetics with reaction mixtures according to Tab. 4.17 (see also 4.13.4)

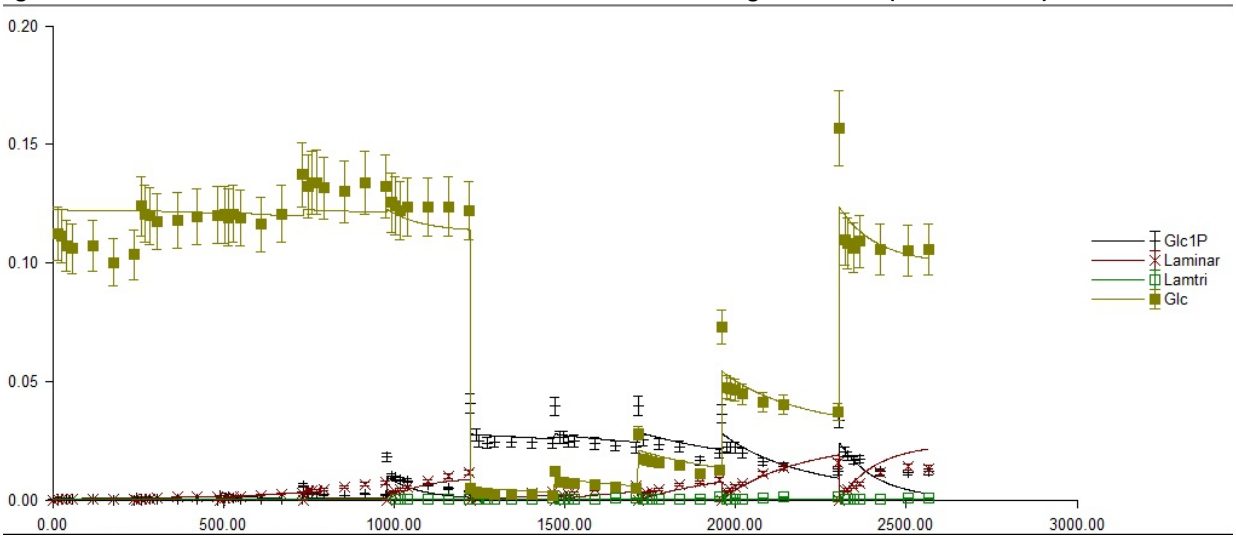

Fig. 11.2 MM3 Models of immobilized LP kinetics in 1.5% Chi-10% PP with reaction mixtures according to Tab. 4.18 (see also 4.13.5)

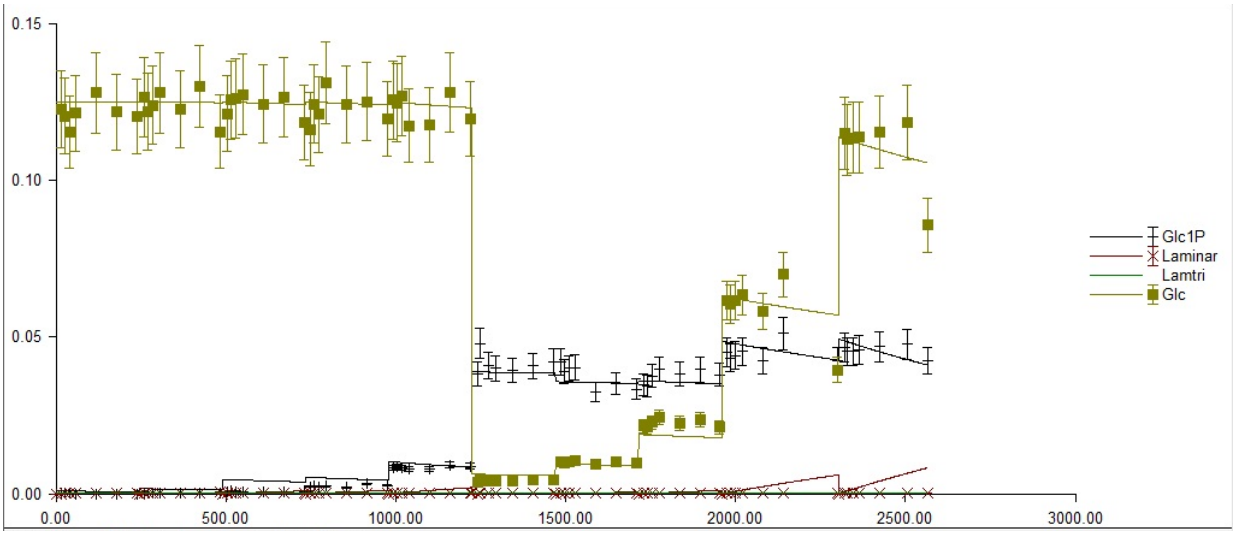

Fig. 11.3 MM3 Models of immobilized LP kinetics in 1.5% Chi-15% PP with reaction mixtures according to Tab. 4.18 (see also 4.13.5)

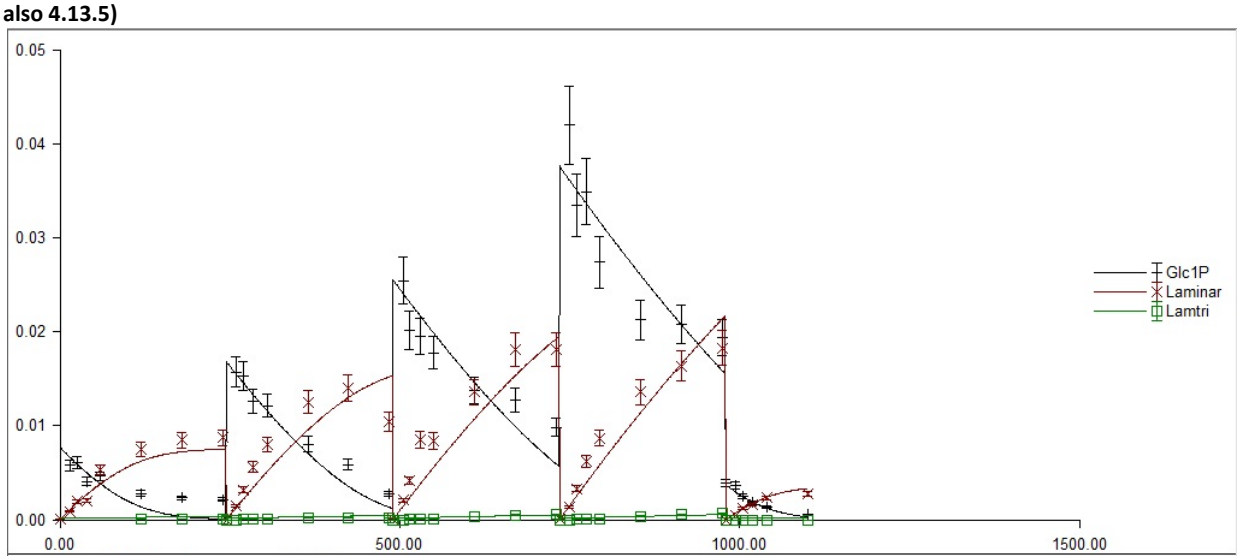

Fig. 11.4 MM3 Models of immobilized LP kinetics in 4% agar with reaction mixtures according to Tab. 4.19 (see also 4.13.6)

11.2 Model diagrams of SP kinetics using ModelMaker3®

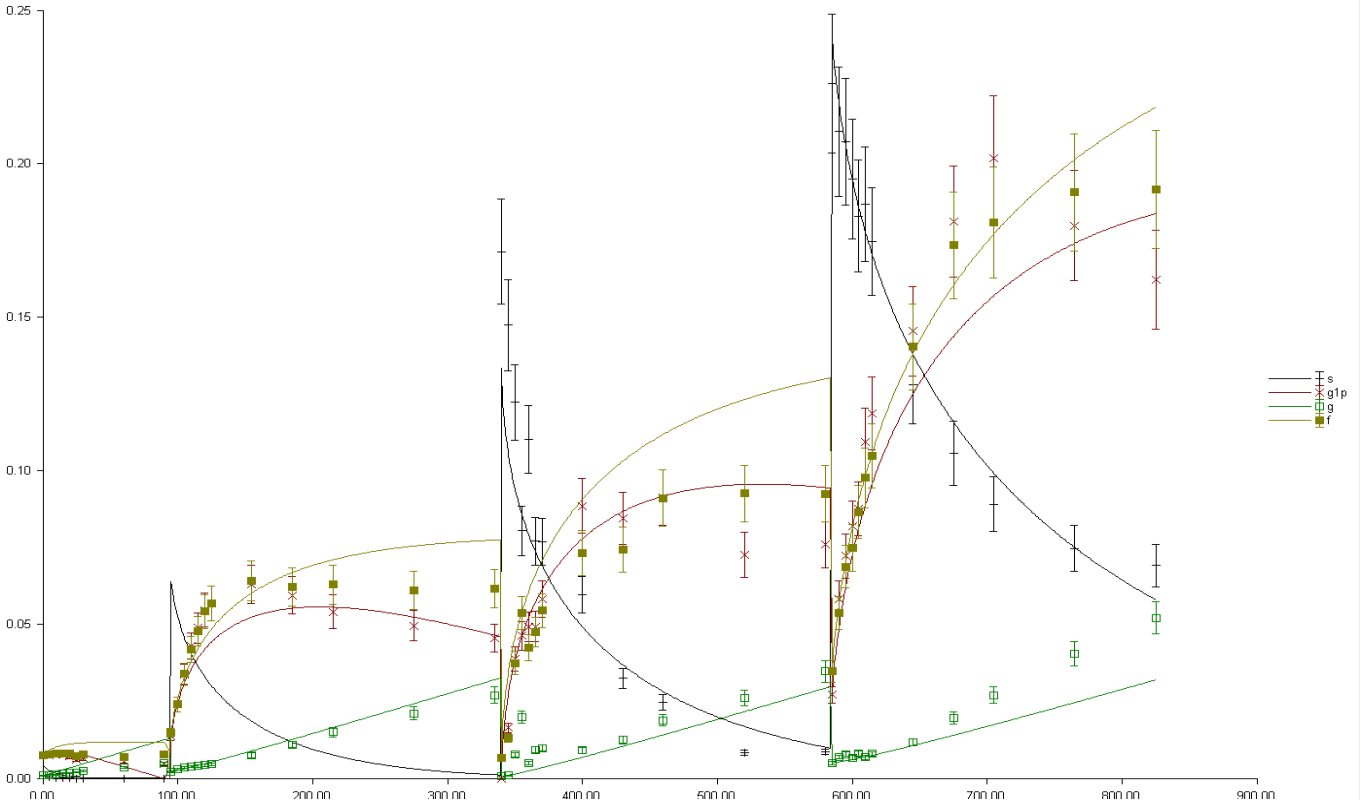

Fig. 11.5 MM3 Models of native SP kinetics with reaction mixtures according to Tab. 4.20 (see also 4.14.1)

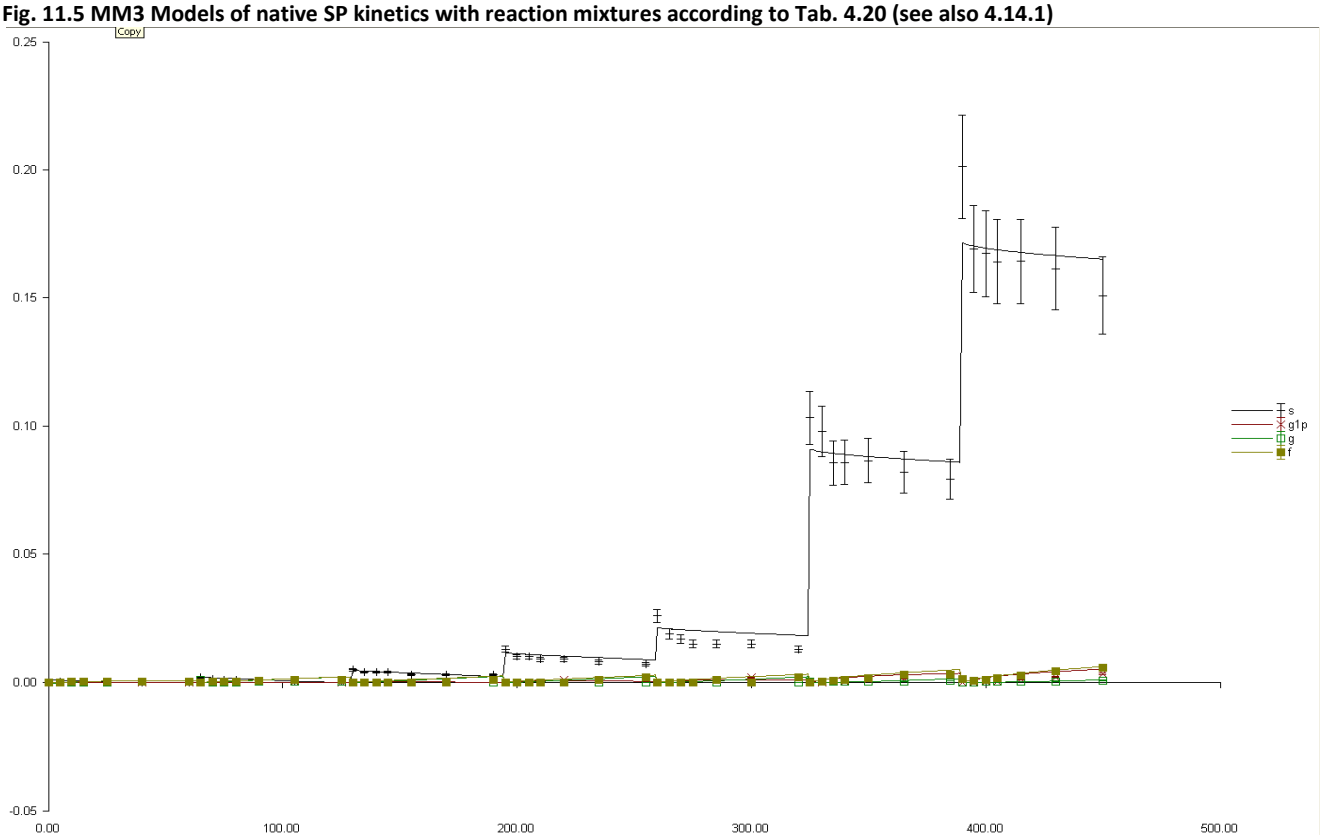

Fig. 11.6 MM3 Models of immobilized SP kinetics in 1.5% Chi-10% PP with reaction mixtures according to Tab. 4.21 (see also 4.14.4)

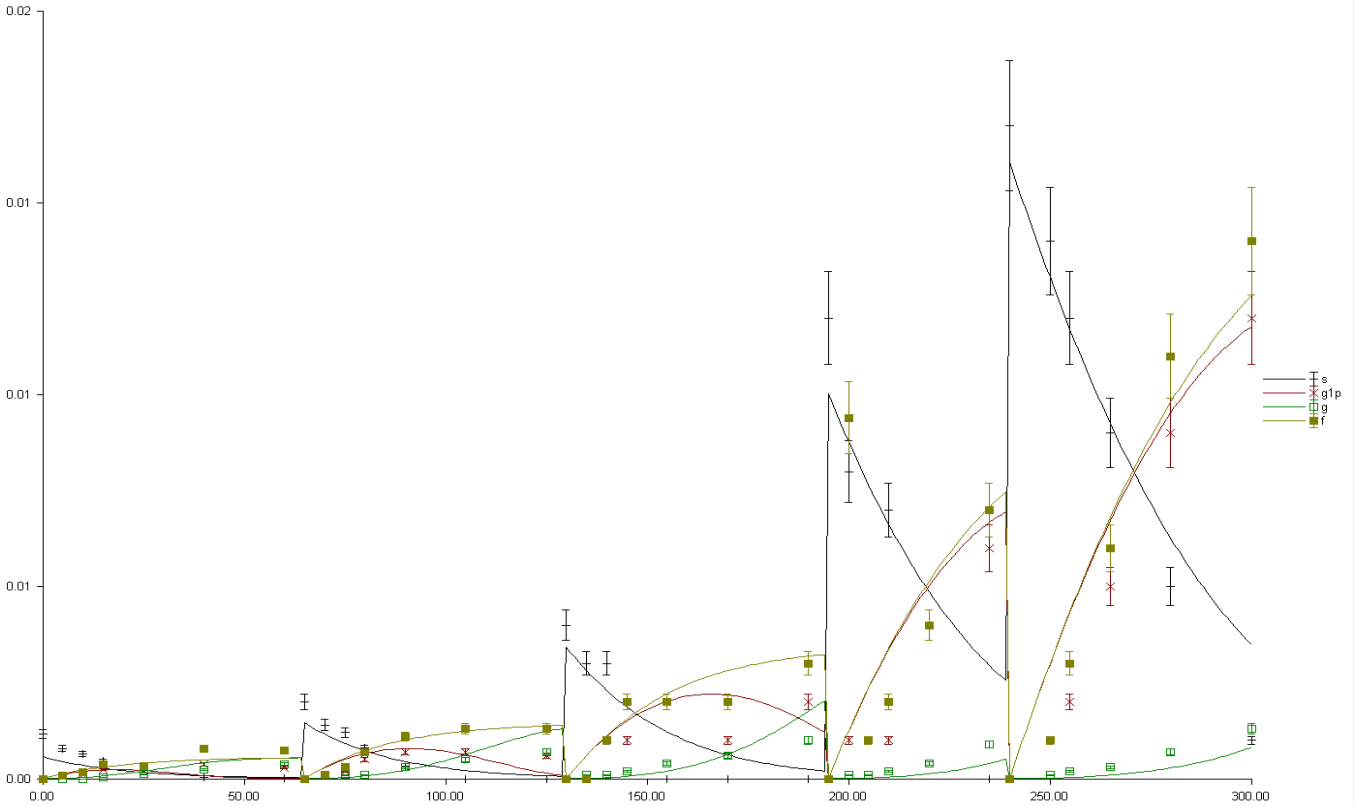

Fig. 11.7 MM3 Models of immobilized SP kinetics in 1.5% Chi-15% PP with reaction mixtures according to Tab. 4.21 (see also 4.14.4)

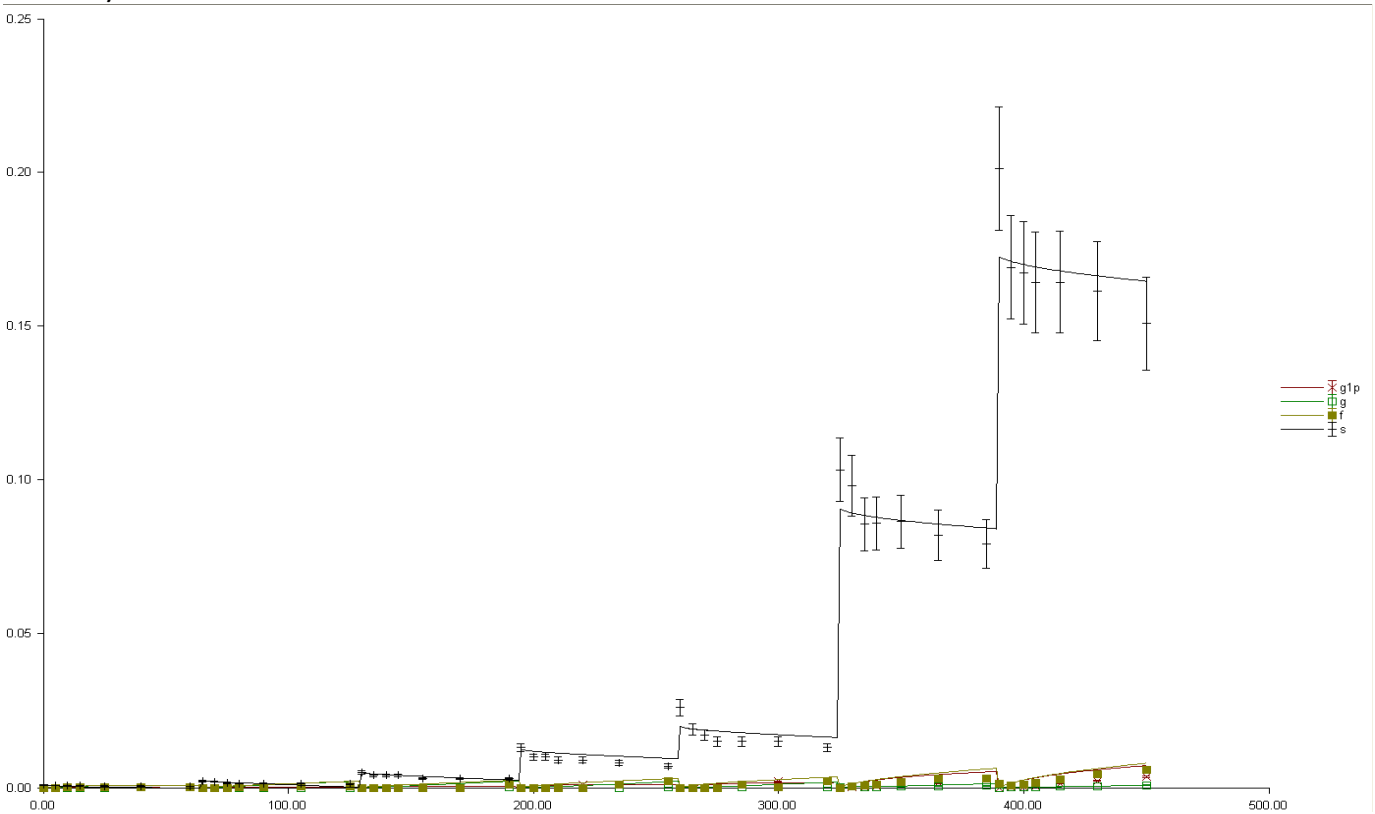

Fig. 11.8 MM3 Models of immobilized SP kinetics in 2% Chi-15% PP with reaction mixtures according to Tab. 4.21 (see also 4.14.4)

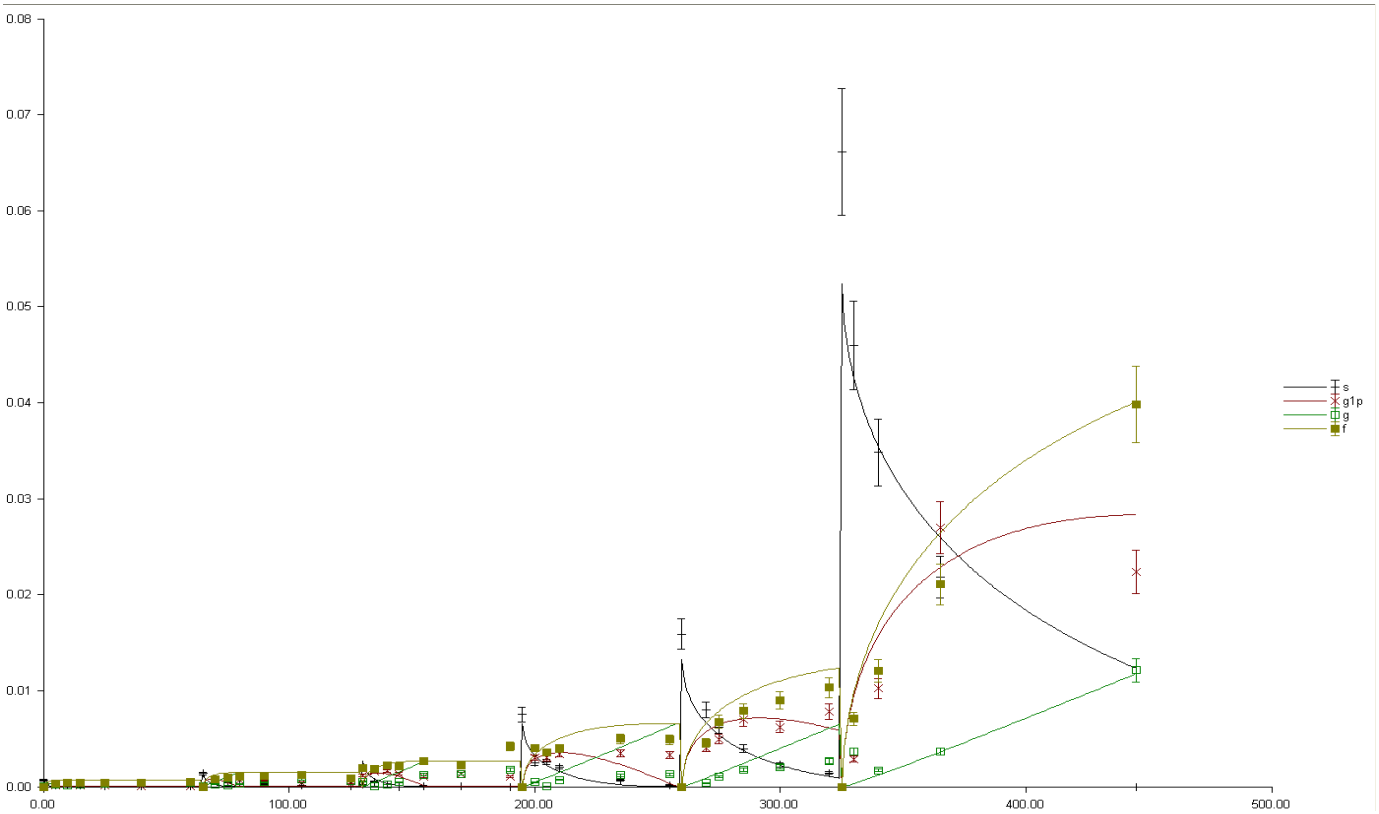

Fig. 11.9 MM3 Models of immobilized SP kinetics in 4% agar with reaction mixtures according to Tab. 4.21 (see also 4.14.5)

11.3 Model diagrams of GI kinetics using ModelMaker3®

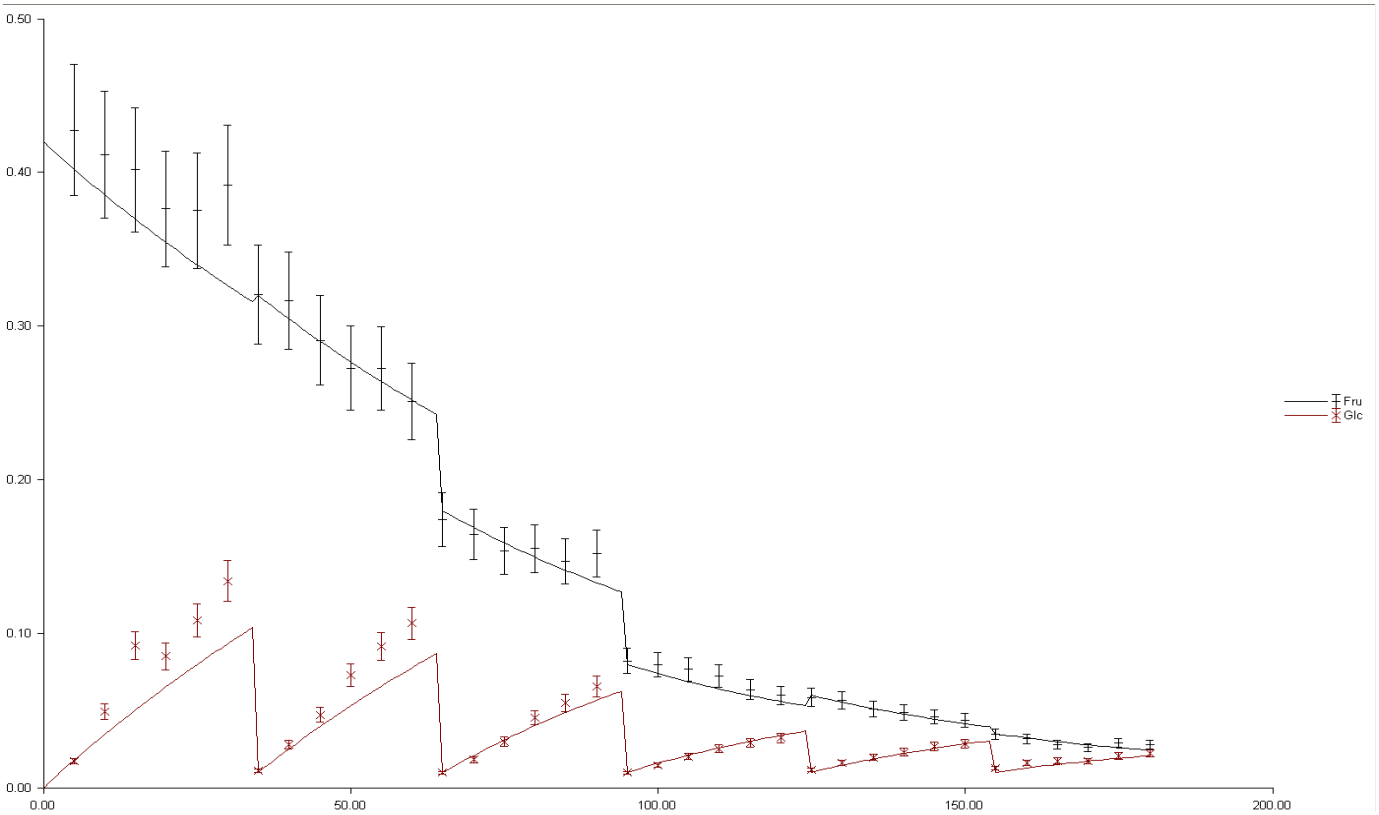

Fig. 11.10 MM3 Models of native GI granule kinetics with reaction mixtures according to Tab. 4.22 (see also 4.15.1)

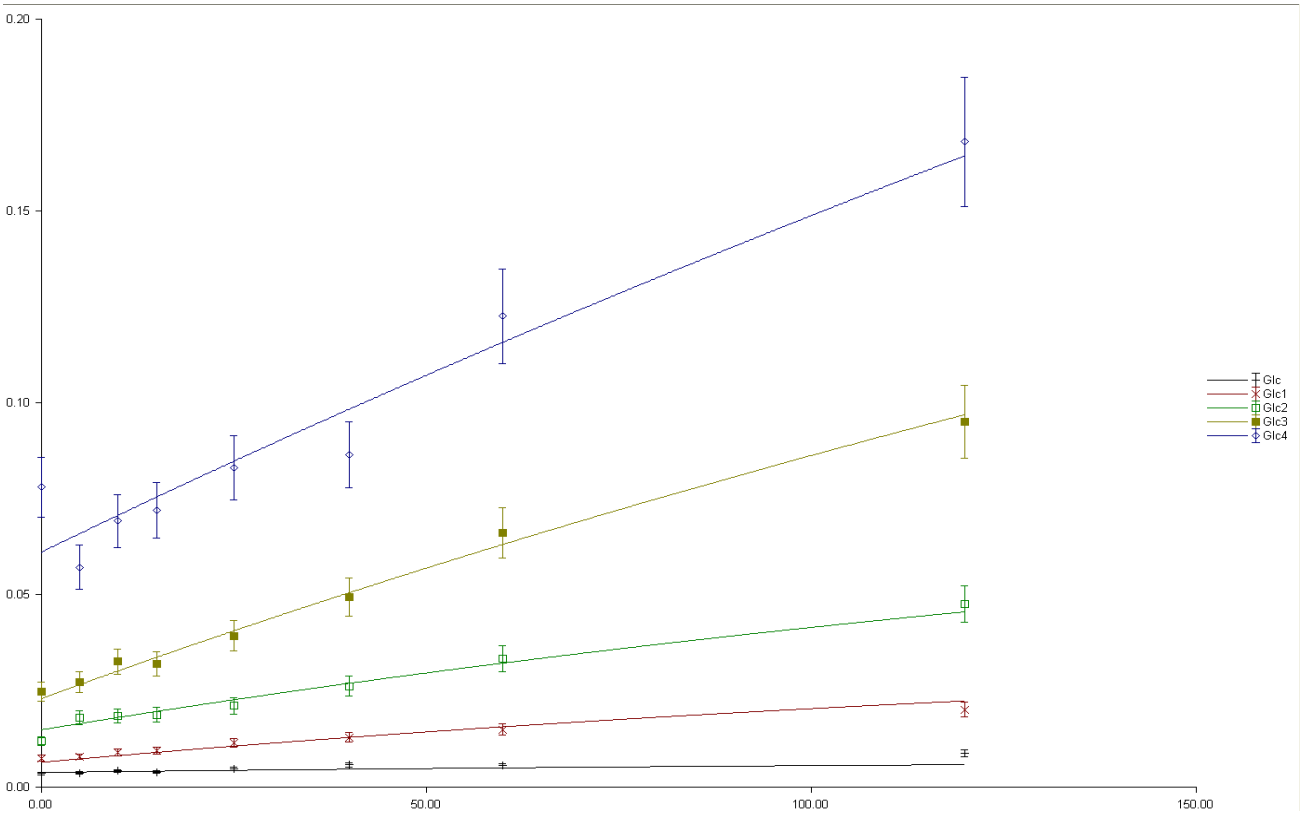

Fig. 11.11 MM3 Models of native GI suspension kinetics with reaction mixtures according to Tab. 4.22 (see also 4.15.1)

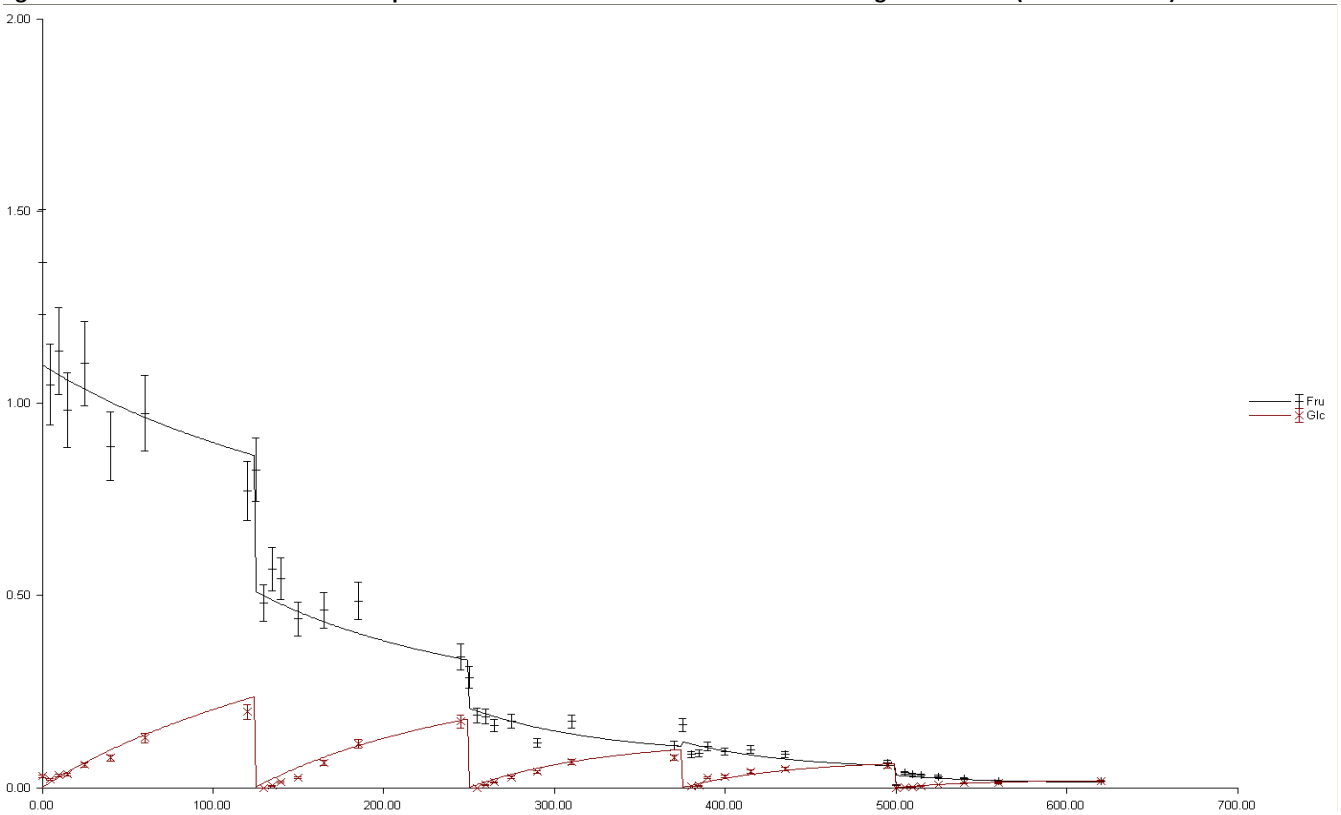

Fig. 11.12 MM3 Models of immobilized GI granules kinetics in 1.5% Chi-10% PP with reaction mixtures according to Tab. 4.23 (see also 4.15.2)

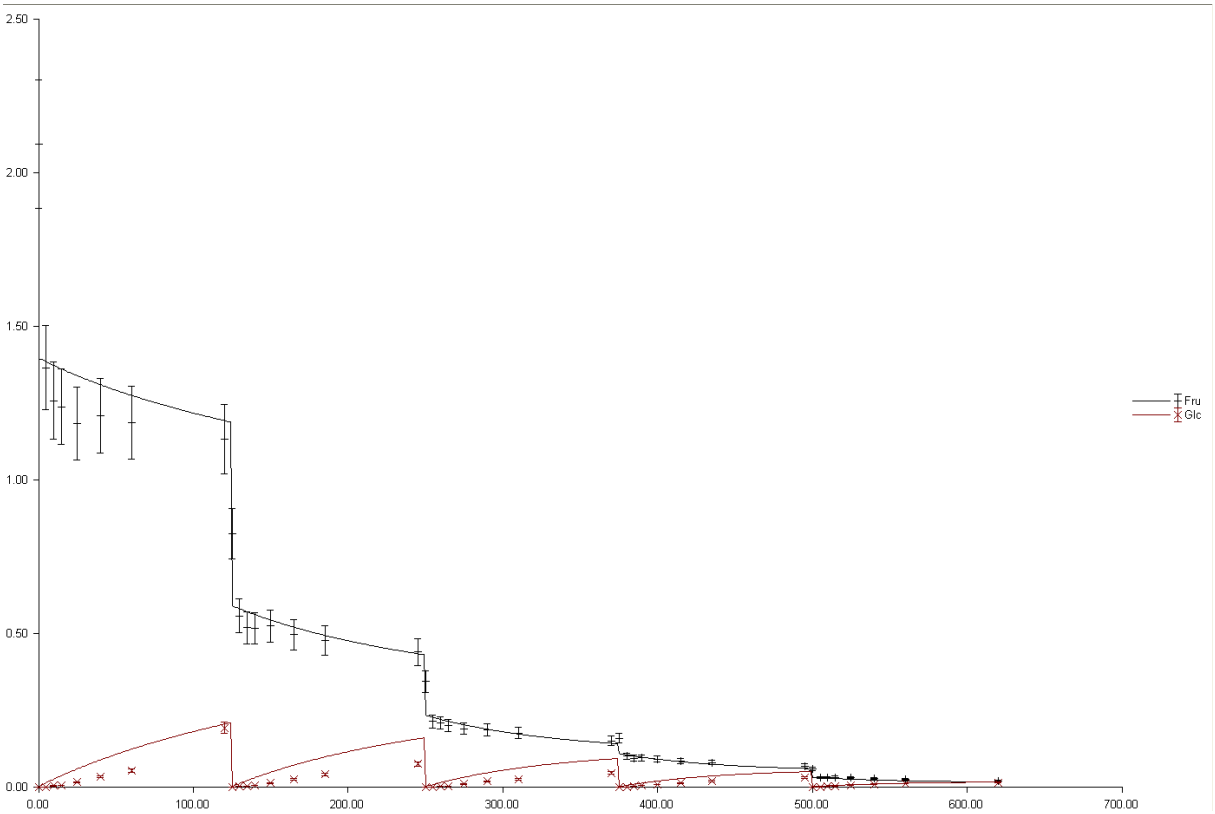

Fig. 11.13 MM3 Models of immobilized GI granules kinetics in 1.5% Chi-15% PP with reaction mixtures according to Tab. 4.23 (see also 4.15.2)

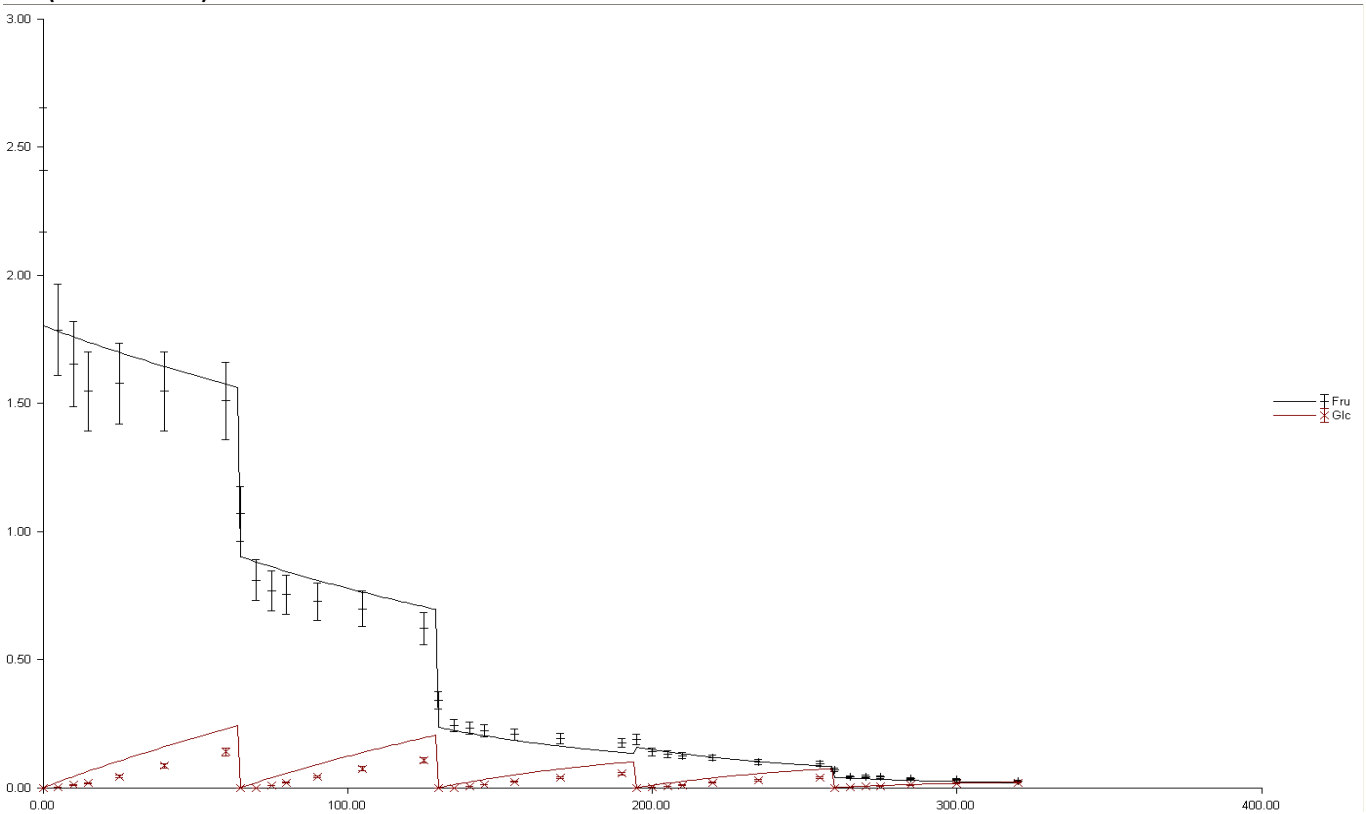

Fig. 11.14 MM3 Models of immobilized GI granules kinetics in 2% Chi-15% PP with reaction mixtures according to Tab. 4.23 (see also 4.15.2)

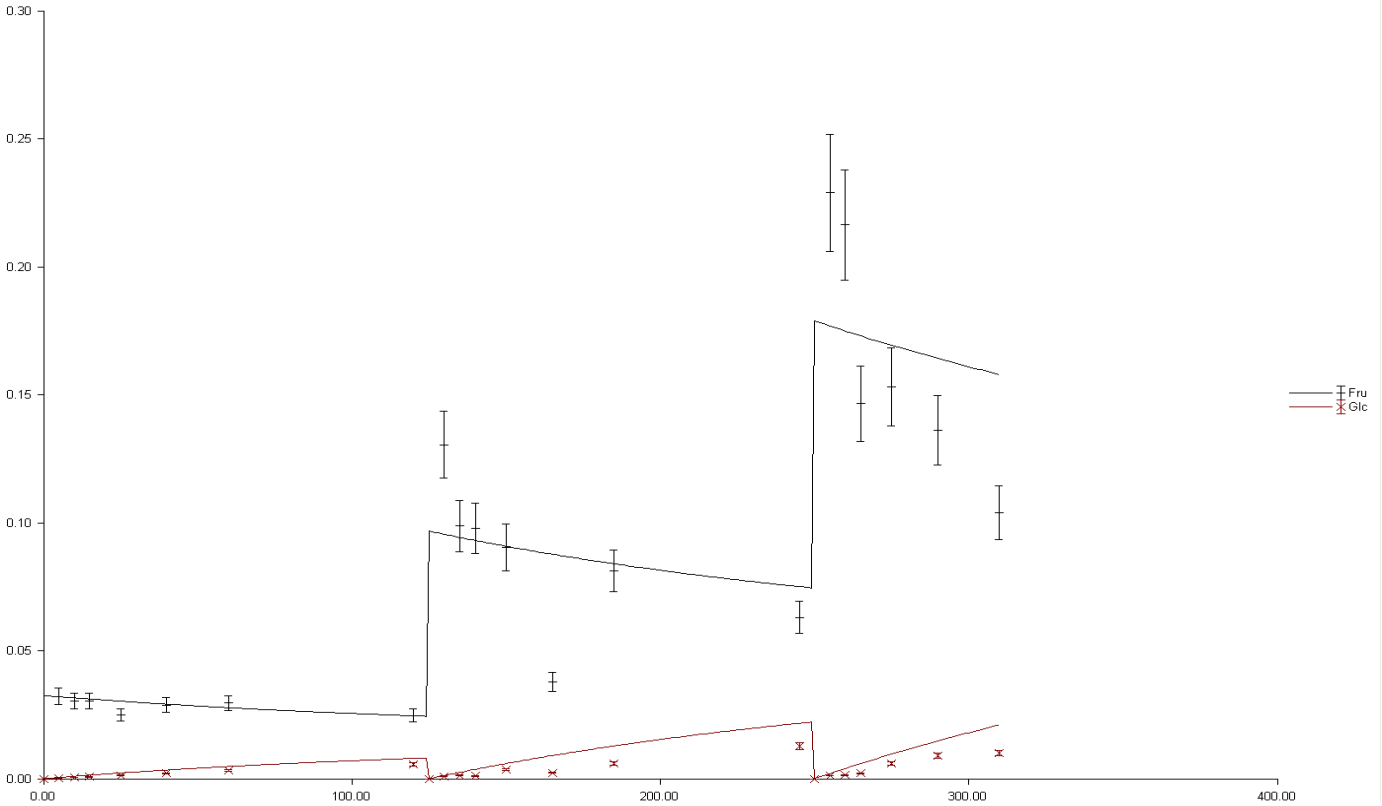

Fig. 11.15 MM3 Models of immobilized GI suspension kinetics in 4% agar with reaction mixtures according to Tab. 4.23 (see also 4.15.2)

11.4 Kinetic modeling of bienzymatic system with ModelMaker3®

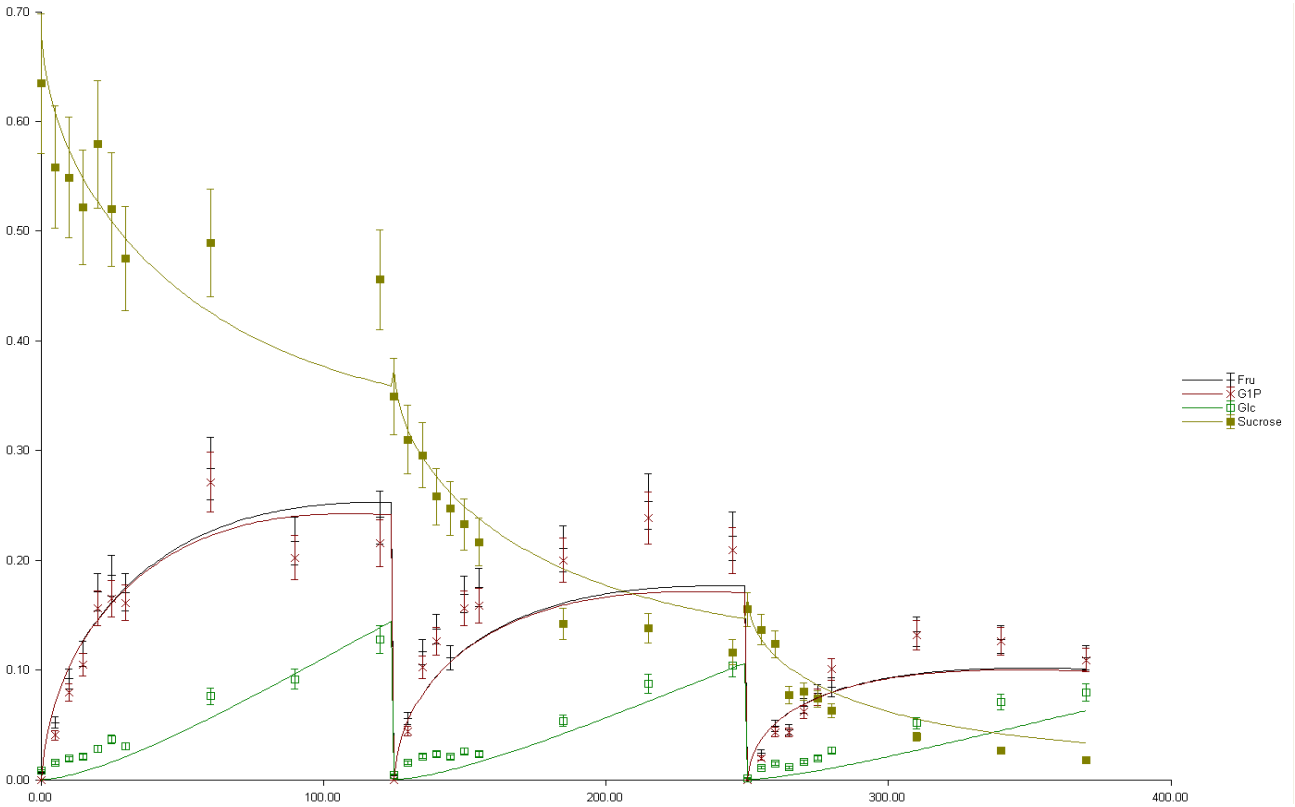

Fig. 11.16 MM3 Models of native bienzymatic system kinetics with reaction mixtures according to 4.16.2